

**TLR AGONISTS ON AUTOIMMUNITY, CANCER AND M1/M2
MACROPHAGE POLARIZATION**

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July 2019

We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Doctor of Philosophy.

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ABSTRACT

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Macrophages play an important role in the initiation of immune responses and the maintenance of immune homeostasis. Alterations in their phenotype, function and activation state have been implicated in the pathogenesis of autoimmune and inflammatory diseases. An increased M1:M2 ratio is associated with the development of several autoimmune diseases including Systemic Lupus Erythematosus (SLE), vasculitis and myositis. Previous work showed that the TLR2/1 agonist PAM3CSK4 (PAM3) could stimulate normal human monocytes to preferentially differentiate into immunosuppressive M2-like rather than inflammatory M1-like macrophages. This work seeks to investigate the ability of PAM3 to induce M2 macrophage differentiation from patient monocytes and evaluate the therapeutic efficacy of PAM3 in a murine model of lupus.

Our findings revealed that patients with indicated autoimmune diseases have a significant increase in monocytes of the inflammatory subtypes coupled with a decrease in non-inflammatory classical monocytes compared to healthy controls. Additionally, in the absence of stimulant patient monocytes differentiated into more M1-like macrophages. Nevertheless, phenotypic analysis of in vitro generated macrophages revealed that, PAM3 stimulation induced M2-like macrophage differentiation without any difference from patient and healthy monocytes. Phenotypic analysis was further supported by the high endocytic abilities and secretion of anti-inflammatory cytokines instead of pro-inflammatory cytokines by PAM3 generated macrophages.

Lupus-prone NZB x NZW F1 mice responded similarly to weekly PAM3 treatment. Upon PAM3 treatment the increased M1:M2 ratio, which was observed in PBS treated group, was decreased to normal levels. The increase in M2 macrophages was accompanied by decreased autoantibody and inflammatory cytokines along with an

increase in anti-inflammatory cytokine production. Moreover, kidney damage was significantly suppressed and M2 macrophages were detected in the kidneys of PAM3 treated group. PAM3 treatment prolonged to survival of NZB/W significantly, at 45 weeks of age %60 of mice were still alive whereas in PBS group only %5 were. Our results indicate that, PAM3 induces immunosuppressive macrophages and thus could represent a novel approach to the therapy of autoimmune diseases.

The second part of this thesis focused on enhancing the immunomodulatory effects of TLR9 ligands, CpG ODN upon encapsulation within liposomes as cancer vaccine adjuvants. Although both D and K ODN are strictly dependent on TLR9, K ODN trigger pDCs to mature and secrete TNF α while D ODN stimulate pDC to produce IFN α . When cells are incubated with a mixture of K and D ODN, K masks the activity of D. The use of both K and D ODN would be of benefit when preparing vaccine adjuvants and for immunotherapy. Our data indicate that simultaneous delivery of D ODN loaded into neutral liposomes plus K ODN loaded into cationic liposomes improved rather than masked IFN α production while continuing to support TNF α by PBMCs. Liposomal encapsulation did not alter the subcellular localization of either class of ODN, but internalization studies revealed that cationic liposome encapsulation slows and reduces the uptake of K ODN whereas neutral encapsulation of D increases their uptake by pDCs. The efficiency of K plus D liposome combinations was examined in a murine tumor vaccine model. The liposome combinations induced pronounced Th1-biased anti-OVA immunity and led to a significant reduction of B16-OVA tumors following inoculation. Our findings, demonstrate that the beneficial features of D and K ODN could be obtained simultaneously by appropriate liposomal formulation, further extending the breadth of CpG ODN-dependent immunotherapy.

Keywords: SLE, vasculitis, myositis, TLR2/1, PAM3, M2 macrophages, NZB/W, TLR9, CpG, Liposomes, Cancer vaccine, Adjuvant

ÖZET

TLR Agonistlerinin Otoimmünite, Kanser ve M1/M2 Makrofaj Polarizasyonundaki Etkileri

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Makrofajlar, immün yanıtların başlamasında ve dengenin korunmasında önemli bir rol oynar. Makrofajların, fenotiplerindeki, fonksiyonlarındaki ve aktivasyon durumlarındaki değişikliklerin, otoimmün ve enflamatuvar hastalıkların patogenezinde etki ettiği gösterilmiştir. M1: M2 makrofaj oranındaki artış, Sistemik Lupus Eritematozus (SLE), vaskülit ve miyozit dahil olmak üzere birçok otoimmün hastalığın gelişimi ile ilişkilendirilmiştir. Daha önceki çalışmalar, TLR2 / 1 agonisti PAM3CSK4'ün (PAM3) normal insan monositlerini, enflamatuvar M1-benzeri makrofajlardan ziyade immünosupresif M2-makrofaj değişimine desteklediği gösterdi. Bu çalışma, PAM3'ün hasta monositlerinden M2 makrofaj farklılaşmasını indükleyebilme yeteneğini araştırmayı ve PAM3'ün bir lupus fare modelinde terapötik etkinliğini değerlendirmeyi amaçlar.

Çalışmalar sonucunda, belirtilen otoimmün hastalıklara sahip kişilerin enflamatuvar tipte monositlerinde bir artış ile birlikte, anti- enflamatuvar olan klasik monositlerde ise bir azalma olduğunu göstermiştir. Ek olarak, bu monositler herhangi bir uyarıcı olmadan kültüre konulduğunda M1 tipi makrofajlara dönüşme eğiliminde oldukları gözlemlendi. PAM3 varlığında ise hasta ve sağlıklı monositleri herhangi bir fark olmadan fenotipik olarak M2 benzeri makrofajlarına değişti. Makrofajların fonksiyonlarını belirlemek için yapılan analizler, fenotipik analizleri destekleyici yonde olup, PAM3 ile geliştirilen makrofajların yüksek endositik yetenekleri olduğu göstermiş ve enflamatuvar sitokinler yerine anti-enflamatuvar sitokin sentezini indüklemişlerdir.

Lupus eğilimli NZB x NZW F1 farelerine haftalık yapılan PAM3 tedavisi sonucunda insan bulgularına benzer sonuçlar elde edildi. PBS enjeksiyonu yapılan farelerde

gözlemlenen M1:M2 makrofaj oranındaki artış, PAM3 tedavisi ise normal seviyelere düştü. M2 makrofajlarında görülen bu artışı, otoantikor ve enflamatuar sitokin seviyelerindeki düşüş eşlik etti. Ayrıca PAM3 tedavisi alan hayvanların böbreklerindeki hasar ise önemli ölçüde bastırıldı. PAM3 tedavisi sonucu 45 haftalık hayvanların %60'ı hala hayatta iken, PBS alan kontrol grubunda bu yüzde %5'e kadar düştü. Sonuçlarımız PAM3'nin monositlerin M2 makrofajlarına değişimini sağladığını ve bu yöntemin otoimmün hastalıklar için bir yeni bir tedavi yöntemi olarak kullanılabileceğini göstermiştir.

Bu tezin ikinci bölümü, TLR9 ligandı olan, CpG ODN'nin, lipozomlar içine yüklenerek kanser aşısı ajanları olarak kullanılmak üzere immünomodülatör etkilerini arttırmaya odaklanmıştır. D tipi ODN, pDC'yi IFN α üretmek için uyarırken, K tipi ODN pDC'lerin mature olmasını destekleyerek TNF α sekrete edilmesine sebep olur. Hücreler K ve D tipi ODN karışımı ile inkübe edildiğinde, K, D'nin aktivitesini maskeleyiği gözlemlenmiştir. Aşı ajanları hazırlanırken aktive ettikleri farklı yolların birleşimi sayesinde hem K, hem de D tipi ODN'nin kullanılmasının yararlı olacağını düşünmekteyiz. Verilerimiz, nötral lipozomlara yüklenen D ODN ve katyonik lipozomlara yüklenen K ODN'nin, PBMC'ler tarafından TNF α 'yı desteklemeye devam ederken IFN α üretimindeki engelin kalkarak, bu sitokinin sentezinde artış olduğunu göstermektedir. Lipozomal yükleme her iki ODN sınıfının da hücresel lokalizasyonunda değişime sebep olmadı, fakat hücre içine alım çalışmaları, katyonik lipozoma yüklenen K ODN'nin hücre alımını yavaşlayıp azaldığını, D'nin nötral lipozoma yüklenmesinin ise pDC'ler tarafından alımını arttırdığını gösterdi. K artı D lipozom kombinasyonlarının etkinliği, bir fare tümör aşı modelinde incelendi. Lipozom kombinasyonları, belirgin Th1 yanlı anti-OVA yanıtını arttırarak, aşılamaı takiben B16-OVA tümörlerinde önemli bir azalmaya neden olmuştur. Bulgularımız, D ve K ODN'nin faydalı özelliklerinin uygun lipozamlara yüklenmesi sonucunda birleştirilerek, immunoterapi alanında faydalı olabileceğini göstermiştir.

Anahtar Kelimeler: SLE, Vaskülit, miyozit, TLR2/1, PAM3, M2 makrofaj, NZB/W, TLR9, CpG ODN, Lipozom, Kanser Aşı Ajanı,



To my precious family...

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Abbreviations

Ab	Antibody
Ag	Antigen
ACUC	Animal Care and Use Committee
APC	Antigen presenting cell
Arg1	Arginase 1
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
Ca	Calcium
CLR	C-type lectin receptors
CpG	Unmethylated cytosine-guanosine motifs
CSF	Colony stimulating factor
CTL	Cytotoxic T cells
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked-immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting

FCS	Fetal calf serum
FDA	US Food and Drug Administration
FITC	Fluorescein isothiocyanate
FIZZ	Found in inflammatory zone 1
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA-DR	Human leukocyte antigen – antigen D related
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMQ	Imiquimod
IMZ	Imidazoquinoline
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
IP-10	Interferon gamma-induced protein 10
IRAK	IL-1 receptor-associated kinase
IRF	Interferon-regulatory factor
I κ B	Inhibitor kappa B
IKK	Inhibitor kappa B kinase
JAK	Janus Kinase
JNK	C-Jun N-terminal kinase
LBP	LPS binding protein
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LN	Lymph node

mAb	Monoclonal antibody
M-CSF	Macrophage colony stimulating factor-1
mDC	Myeloid dendritic cell
MeOH	Methanol
MFI	Mean fluorescence intensity
MAPK	Mitogen activated protein
MD-2	Myeloid differentiation factor 2
MDA5	Melanoma Differentiation-Associated protein 5
MDP	Macrophage and DC precursor
MDSC	Myeloid-derived suppressor cells
MHC	Major Histocompatibility Complex
MIP1 α	Macrophage Inflammatory Protein 1 alpha
MMR	Macrophage mannose receptor
MMTV	Mouse mammary tumor virus
moDC	Monocyte-derived dendritic cells
MPLA	Monophosphoryl lipid A
MPS	Mononuclear phagocyte system
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
NCI	National Cancer Institute
NF- κ B	Nuclear factor-kappa B
NCI	National Cancer Institute
NK	Natural killer cell
NLR	Nucleotide-binding oligomerization domain like receptors
NO	Nitric oxide
NOD	Nucleotide-binding Oligomerization Domain

ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cells
PI3K	Phosphoinositide 3-kinase
poly(I:C)	Polyinosinic-polycytidylic acid
PNPP	p-nitrophenyl phosphate
PO-	Phosphodiester backbone
PRRs	Pattern recognition receptors
PS-	Phosphorothioate backbone
PSA	Polysaccharide A
RIG	Retinoic acid-inducible gene
RLR	RIG-I-Like Receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
s.c.	Subcutaneous
siRNA	Small interfering RNA
SR	Scavenger receptor
ssDNA	Single stranded DNA
ssRNA	Single-stranded RNA
STAT	Signal transducers and activators of transcription
TAK	TGF- β activated kinase
TAM	Tumor associated macrophage
TBK	TANK-binding kinase
TCR	T-cell receptor

Th	T-helper
TIR	Toll/IL-1 receptor
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing adapter-inducing IFN- β
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor
VLP	Virus-like particle

Chapter 1

Introduction

1.1 The Immune System

Immune system is a powerful and efficient defense mechanism consisting of effector cells, soluble factors and physical barriers that protect the host from pathogenic non-self [1]. The immune system consists of two interacting elements: innate and adaptive.

The innate immune system represents the first and most rapid line of defense against pathogens after the first physical barrier; dermis. It evolved about 700 million years ago [2, 3] and has elements that are present even in lower level organisms such as plants and invertebrates. The innate immune system includes receptors that recognize common components shared by a variety of bacteria, fungi and viruses collectively referred to as “pattern recognition receptors” (PRRs) [1]. Cells of the innate immune system include phagocytes, dendritic cells and innate lymphoid cells [4]. Neutrophils and macrophages are phagocytes that participate in the recognition and ingestion of microbes. They also release cytokines and chemokines that recruit other cells to the site of infection [5]. Dendritic cells play an important role in the presentation of antigens to cells of adaptive immunity. Upon activation, DCs migrate to lymph nodes where they interact with T cells and present microbial peptides [6].

Adaptive (acquired) immunity provides a broader and more finely tuned response that differentiates between self and non-self-antigens. Innate immune signals are essential for the activation of adaptive immunity. Adaptive immunity is initiated through the interaction of antigen presenting cells with T and B lymphocytes [7]. Cells of the adaptive immune system are essential for the initiation of pathogen specific immune responses and generation of immunologic memory. Innate immune

system cells migrate to lymphoid organs upon recognition of pathogens where they present antigens to lymphocytes. T cells are important for the generation of cell-mediated immunity whereas B cells are required for the secretion of antibodies as a part of the humoral immune response [8].

1.2 The Innate Immune System

1.2.1 Pattern Recognition Receptors

As noted above, cells of the innate immune system express PRRs that facilitate the recognition of pathogens [3]. In addition to recognizing elements present in various bacteria and viruses, PRRs also recognize danger associated molecular patterns (DAMPs) released from stressed, damaged, necrotic or apoptotic cells [9].

Different PRRs are expressed either on the cell's surface membrane or within specific intracellular compartments [10]. Nucleic acid sensing PRRs such as retinoic acid inducible gene-1 (RIG-1) like receptors (RLRs) and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) recognize viral nucleic acids and peptidoglycan-derived products of virus and bacteria and are found in the cytoplasm [11, 12].

PRRs trigger macrophages and dendritic cells to upregulate transcription of genes involved in inflammatory responses [13]. C-type lectin receptors (CLRs), scavenger receptors and Toll-like receptors (TLRs) can be found either on cell surface or within endosomal membranes. Dectin-1, Dectin-2, mannose receptor and DC-SIGN are part of the CLRs and can recognize carbohydrate groups on the surface of bacteria and fungi. Scavenger receptors are able to recognize DAMPs and PAMPs that includes endogenous proteins, bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA) [14].

TLRs are the most widely studied and characterized family of PRRs. They can recognize a wide range of microbial components including cell wall products, flagellin, dsDNA, ssRNA and unmethylated CpG DNA motifs [15].

1.2.2 Toll-like receptors

Toll-like receptors are found both in vertebrates and invertebrates. They were initially described in *Drosophila melanogaster* and their mammalian homologs were identified in 1997 [16-18].

TLRs are members of the Toll-interleukin 1 receptor (IL-1R) family, containing leucine-rich repeat motifs which mediate the recognition of “pathogen associated molecular patterns” or PAMPs, a transmembrane component and an intracellular Toll-IL-1 receptor (TIR) domain that activates downstream signaling pathways. In human ten TLRs (TLR1-10) and in mice twelve (TLR1-9 and TLR11-13) has been described [19]

Two major pathways are activated in TLR signaling; the myeloid differentiation primary response gene 88 (MyD88) and the TIR-domain containing-adaptor inducing interferon β (TRIF) dependent pathways. Almost all TLRs use the MyD88-dependent pathway an exception is TLR3 which use TRIF-dependent and TLR4 can use both pathways [20]. Depending on their cellular localization TLRs can be divided into two subgroups; surface and intracellular TLRs [21]. TLRs 1,2,4,5,6 and 10 are found on the cell surface and generally recognize bacterial products. On the other hand, TLRs 3,7,8,9,10,11,12 and 13 are responsible for the recognition of nucleic acids and are expressed intracellularly [19, 21, 22] (Figure 1.1).

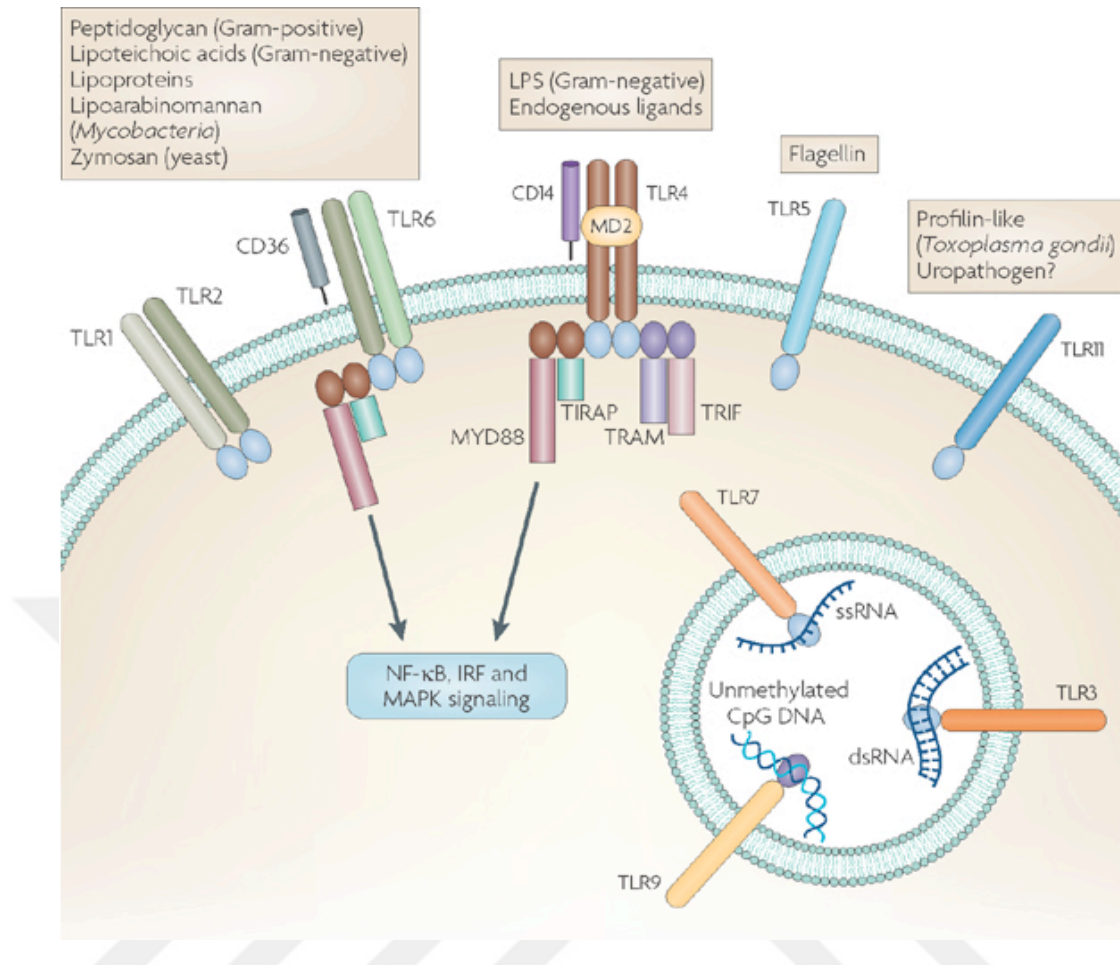


Figure 1.1 Schematic distribution of human TLRs and PAMPs recognized by them [20].

Upon activation of TLRs, IL-1 receptor associated kinase IRAK4 is recruited by MyD88 adaptor protein which leads to the phosphorylation of IRAK2 and IRAK1 [23, 24]. Phosphorylated IRAK1/2 activates transforming growth factor- β -activated kinase 1 (TAK1) by interacting with tumor necrosis factor-receptor-associated factor (TRAF6). NF κ B is recruited to the nucleus by the phosphorylation of I κ B kinase (IKK) by TAK1. TAK1 can also activate other signaling pathways including JNK (c-jun N-terminal kinase), ERK (extracellular-signal-regulated kinase) and p38. After transcription factors are recruited to the nucleus, they induce pro-inflammatory cytokines including IL-1 and TNF α . TLR7 and 9 activation results in type-I IFN release using IRF7[20, 25, 26].

1.2.3 Surface TLRs

TLRs 1,2,4,5,6 and 10 are found on the cell surface and can recognize both surface PAMPs and DAMPs. TLR2 recognizes various microbial components including lipoproteins, lipopeptides, peptidoglycans and lipoteichoic acid from gram-negative and gram-positive bacteria, lipoarabinomannan from mycobacteria, glycosylphosphatidylinositol anchors from *Trypanosoma cruzii*, a phenol-soluble modulins from *Staphylococcus epidermis*, zymosan from fungi and glycolipids from *Treponema maltophilum* [27]. Some of this diversity is explained by the ability of TLR2 to form heterodimers with TLRs 1,6 and 10 and certain non-TLR molecules (e.g. CD36, Dectin-1). Specific TLR2 heterodimers recognize different PAMPs [21]. The TLR2/1 heterodimer binds to triacyl lipopeptides (PAM3CSK4 is the synthetic analogue) while TLR2/6 recognizes diacyl lipopeptides (PAM2CSK4 is the synthetic analogue) [28-32].

TLR4 is essential in the recognition of lipopolysaccharide (LPS) present on gram negative bacteria and other PAMPs including respiratory syncytial virus (RSV) proteins, mouse mammary tumor virus (MMTV) and pneumolysin from gram positive *S. pneumoniae* [33-35]. Recognition of LPS is not a result of a direct binding. LPS first binds to LPS binding protein (LBP) which transfers LPS onto CD14 a co-receptor. TLR4 can also recognize DAMPs such as hyaluron, fibrinogen, Hsp60,70 and HMGB-1 [36-40]. TLR4 is the only TLR that can initiate both MyD88 and TRIF- dependent pathways. IL-6, IL-12 and TNF α are induced by the MyD88-dependent pathway whereas expression of type I IFNs are upregulated by the TRIF-dependent pathway upon activation of TLR4.

TLR5 recognizes flagellin, a protein component of bacterial flagella which is essential for bacterial motility[41, 42]. The structure of flagellin is highly conserved and found both in gram negative and positive bacteria [43]. Most epithelial cells, monocytes and DCs (particularly in the small intestine) express TLR5.

TLR10 is a pseudogene in mouse, but in humans TLR10 can collaborate with TLR2 to recognize ligands from listeria and also involved in the recognition of influenza A virus[19, 44, 45].

1.2.4 Intracellular TLRs

Unstimulated TLRs 3,7,8 and 9 are located in the ER. Upon interaction with their cognate ligand, they are transferred to the Golgi and endosomal vesicles. TLR3 recognizes double stranded RNA (dsRNA) and single stranded RNA (ssRNA) from virus as well as synthetic polyinosinic-polycytidylic acid (poly I:C). Activation of TLR3 stimulates type I IFN α and inflammatory cytokine production through a TRIF-dependent pathway [10, 21].

TLRs 7 and 8 are closely related with both recognizing guanosine and uridine rich ssRNA of viral or bacterial origin. Synthetic ligands for TLR7/8 include imidazoquinolines [46]. TLR7 is generally expressed predominantly on plasmacytoid DCs (pDCs) and to some extent on monocytes, macrophages and B cells whereas TLR8 is expressed by many immune cells.

TLR9 recognizes ssDNA containing unmethylated CpG motifs (CpG DNA) which are present abundantly in bacterial and viral genomes [47, 48]. TLR9 does not recognize methylated motifs thus mammalian DNA does not activate TLR9 due to the high frequency of methylated CpG dimers it contains [49]. Mouse and human cells differ in their expression of TLR9. B cells, monocyte, macrophages and DC can express TLR9 in mice whereas in humans only pDCs and B cell do [50]. Activation of TLR9 induces a Th1 biased adaptive immune response that improves antigen presentation [51-53]. Synthetic oligonucleotides containing CpG motifs can mimic bacterial DNA and are recognized by TLR9.

TLR11 recognizes flagellin or unknown proteinaceous component of uropathogenic *E. Coli* (UPEC) along with profilin-like molecule from *Toxoplasma gondii* [19, 54]. TLR12 which is predominantly expressed by myeloid cells is very similar to TLR11, forms homodimer or heterodimers with TLR11 and recognizes *T. Gondii* profilin [19, 55]. TLR13 recognizes bacterial 23S rRNA and vesicular stomatitis virus components [19, 56].

1.2.5 CpG Oligodeoxynucleotides

CpG oligodeoxynucleotides (CpG ODN) are synthetic oligodeoxynucleotides that contain unmethylated CpG motifs that mimic bacterial CpG DNA. The immunostimulatory effects of CpG ODNs were first reported in 1992 [57]. These synthetic CpG ODNs contain unmethylated CpG dinucleotides flanked by two 5' purines (Pu) and 3' pyrimidines (Py). Using CpG ODN as adjuvants can enhance the immunostimulatory efficacy of vaccines both in mice and humans [58]. Three main classes of CpG ODNs have been described with structural differences that induces different immune responses [59].

D-type CpG ODN (also referred as type A) have a mixed phosphodiester/phosphorothioate (PO/PS) backbone, contain a single CpG sequence and a poly G tail at the 3' end. Due to this poly G tail, D-type CpG ODN forms G-tetrads and ODN clusters [60]. Upon internalization D-type CpG ODNs localize to early endosomes where they are recognized by TLR9. D-type CpG ODN induce secretion of IFN α by the interaction of MyD88 and IRF7. Because of their tendency to aggregate, there have been batch-to-batch variations in stimulatory activity observed in material produced for clinical testing which caused concern over reproducibility for clinical use.

K-type CpG ODNs (also known as type B) have a complete phosphorothioate (PS) backbone and can contain multiple CpG motifs. The PS backbone decreases their susceptibility to DNase digestion and increases their half-life in vivo [60]. Unlike D-type CpG, K ODN do not have a poly-G tail and thus do not form aggregates. K-type CpG ODN are quite stable and have been widely studied in pre-clinical and clinical settings [58, 61]. Upon internalization, K-type CpG ODN rapidly localize to late endosome, activate NF κ B and pro-inflammatory cytokines including TNF α , IL-6, IL-12 and IFN γ [62-64]. K-type CpG also promote the maturation and activation of pDC, but do not induce secretion of type I IFNs (in contrast to D-type ODN). K ODN also induce B cells to proliferate and secrete IgM [63]. Clinical trials using K-type CpG as vaccine adjuvants and for the treatment of diseases including allergy and cancer are ongoing [52, 65-68].

Third type of CpG ODN is the C-type which have properties of both D and K ODNs [61]. C type have a full PS-backbone and palindromic CpG motifs at the 3' end which leads them to form duplexes [60, 69]. C-type CpG ODN stimulate B cells to secrete IL-6 and IFN α from pDCs to a lesser degree than D-type CpG [70]. Other types of CpG ODNs that do not fit in any other class described above includes P-type and Y-type CpG ODNs. P-type CpG ODNs contain two palindromic sequences and stimulate both B cells and pDC to secrete IFN α [59]. Y-type CpG ODN primarily induce the secretion of TNF α and IL-6 [71].

1.2.6 Delivery of CpG ODNs with Liposomes

CpG ODNs have strong immunomodulatory effects. However, when administered in their natural form, they are susceptible to degradation by nucleases in vivo. It is well established that encapsulation of CpG ODNs into liposomes improves their uptake by macrophages and DCs and increases their immunostimulatory effects [72, 73].

Liposomes are small vesicles consisting of cholesterol and natural non-toxic lipids. They can be synthesized in various sizes. Large multilamellar vesicles (LMV) are from 500 nm to 5 μ m in size. Small unilamellar vesicles (SUV) consist of a single bilayer and are approximately 100 nm in size whereas large unilamellar vesicles are from 200 – 800 nm in diameter [74]. Liposomes are considered to be promising delivery systems. Both amphiphilic and lipophilic molecules can be loaded into liposomes. The bilayer defines the rigidity and fluidity of the liposome and according to the lipid composition they can be in various sizes and have different surface charges. They are currently used extensively in the cosmetic and pharmaceutical industries [75].

Liposomal encapsulation of CpG ODNs protects them from nuclease degradation and prolongs their circulation time thus enhancing their immunostimulatory activities [76]. Liposomal CpG ODNs can be used as adjuvants for anti-viral, anti-bacterial and anti-cancer vaccines or as immunotherapeutic agents. In addition to protecting their cargo, liposome encapsulation also facilitates the uptake of antigens by APC which enhances antigen recognition and vaccine specific immune responses

[77]. For example, encapsulation of CpG plus OVA antigen enhanced Th1 and cell mediated immune responses against OVA [78].

1.3 Myeloid Cells

1.3.1 Monocytes

The mononuclear phagocyte system (MPS) is important for anti-pathogen defense and help to orchestrate immune responses to eliminate foreign agents [79, 80]. MPS includes circulating monocytes, resident macrophages and dendritic cells [81]. Monocytes can be found in blood, bone marrow and spleen. They constitute 4% and 10% of total leukocytes in mice and human respectively. Monocytes derive from hematologic precursors in the bone marrow and then enter the blood circulation. If they are not recruited into a tissue after 1-2 days in circulation they die and are removed. Colony stimulating factor (CSF-1 or MCSF) produced by stromal cells regulates the development, maintenance, proliferation, differentiation and function of monocytes [82]. Granulocyte macrophage colony-stimulating factor (GM-CSF) is another factor involved in the development of monocytes in inflammatory states [83].

Studies suggest that monocytes are innate effector cells that can remove pathogens, produce reactive oxygen species and secrete inflammatory cytokines. In some cases, they also trigger T cell responses [84]. Along with their role in the innate immune responses they form a reservoir for the generation of macrophages and DCs.

In recent years, three functional subsets of human monocytes have been identified based on the expression of the surface markers CD14 and CD16. The dominant population of human monocytes (90% of the total) express high levels of CD14 but no CD16 and are termed 'classical monocytes'. The remaining 10% of human monocytes are divided into an 'intermediate' subset that is CD16 low but CD14 high and the 'non-classical subset' with high CD16 but lower CD14 expression [85]. A number of studies have delineated the function of these monocyte subsets. Classical monocytes are highly phagocytic but non-inflammatory whereas non-classical monocytes manifest strong inflammatory functions. Intermediate monocytes are a transitional subset with both phagocytic and inflammatory functions [86].

1.3.2 Macrophages

Macrophages were defined by Metchnikoff by their ability to consume pathogens. He identified two different cell populations with phagocytic activity and named them 'big eater' in Greek; microphages (neutrophils) and macrophages [87]. They are a major part of the immune system with essential roles in tissue homeostasis, regulation of metabolism, wound healing and the pathogenesis of certain inflammatory diseases [88-91]. It was believed that all macrophages were derived from circulating monocytes. However, recent studies show that tissue resident macrophages are derived from the yolk sac or fetal liver [92]. These macrophages have high self-renewal capacities and their frequencies are stable over time [93].

During inflammatory reactions monocytes can mature into macrophages. Depending on the stimulus, monocytes can differentiate into a wide range of macrophages with distinctive functions. The polarization of macrophages is a highly dynamic process. A recent classification between two major populations was made by mirroring the Th1/Th2 polarization concept: classically activated M1-like (inflammatory) and alternatively activated M2-like (suppressive) macrophages [80].

1.3.2.1 M1-like Macrophages

M1 macrophages are evoked by the stimulation of TLR ligands including bacterial lipopolysaccharide (LPS), lipoteichoic acid (LTA), granulocyte-macrophage colony-stimulating factor (GM-CSF) or by the combination of LPS and IFN γ [94-96]. M1 stimuli activate interferon regulatory factors (IRF) and STAT1 as well. IRFs induce further secretion of pro-inflammatory cytokines such as IL12, IL23 and TNF α [97]. Activation of the NF-KB pathway plays a major role in regulating pro-inflammatory cytokine secretion as well. M1 macrophages help protect the host by directly killing pathogens. Secretion of reactive oxygen species (ROS) and nitrogen radicals (NO) increase their pathogen killing abilities [98]. In order to eliminate pathogens, they also induce the generation of Th1 biased immune response by producing pro-inflammatory molecules such as IL-1 β , IL-6, IL-12, IFN γ and TNF α and expression of co-stimulatory molecules including CD80, CD86 and MHCII [99-101]. Increased expression of surface marker expressions on M1 macrophages related with co-stimulation (ie CD80, CD86 and MHCII) increase macrophages' ability to present

antigens to T cells. Additionally, they secrete chemoattractant that drives recruitment of NK and Th1 T cells at the sites of infection [102].

1.3.2.2 M2-like Macrophages

M2 macrophages exhibit anti-inflammatory features, are endocytic, and play a role in wound healing [103]. M2 macrophages express more arginase and less iNOS than M1 macrophages do and thus produce less NO [104]. Macrophage colony stimulating factor (MCSF) treatment of human monocytes results in a population of macrophages termed M2-like macrophages. MCSF induced M2 macrophages secrete high levels of IL-10 and fail to activate Th1 cells. Other stimulants that results in the generation of M2 macrophages are IL-4, IL-12, PGE₂, IL-10 and/or MCSF or stimuli release by fungi and parasites [95, 96, 104]. IL-4 and IL-13, which are produced by mast cells, TH2 cells, eosinophils, basophils or by macrophages during infection, are considered the main drivers of M2 type macrophage differentiation IL-4 also induces the expansion of tissue resident macrophages [105].

Phenotypic markers of M2 macrophages include FIZZ1, Tm1, macrophage mannose receptor (MMR), CD206 C-type lectin receptor, DC-SIGN (CD209), scavenger receptors SRA (CD204) and CD163. The expression of these markers is increased upon IL-4 and/or IL-13 stimulation [104, 106-109]. In addition to surface markers, the production of anti-inflammatory cytokines (ie TGF- β , IL10, CCL18 and IL1R α) are classical features of IL-4 generated macrophages [110].

There is evidence that a reduction in the number or activity of M2 macrophages (or a relative increase in M1 macrophages number/activity play a role in the development of inflammatory and autoimmune diseases [111]. Thus, therapies aimed at reducing M1 macrophage or increase M2 macrophage frequencies have great potential in the treatment of autoimmune diseases [111, 112].

1.3.2.3 Tumor Associated macrophages

Tumor associated macrophages (TAMs) have crucial roles in tumor cell migration, invasion, intravasation and tumor growth [89, 113, 114]. All these events are required for tumors to become metastatic. TAMs originate from bone marrow

derived monocytes and are recruited to tumor sites by chemoattractant molecules secreted by tumor cells (ie CCL2, VEGF, or MCSF).

Once monocytes are recruited into a tumor, chemokines and growth factors secreted by stromal and tumor cells in the tumor environment promote their maturation. Increased production of TGF- β and CSF-1 in the tumor microenvironment induce TAMs to display phenotypic and functional characteristics of M2 macrophages [115, 116]. They express high levels of anti-inflammatory cytokines, scavenger receptors and angiogenic factors that are more similar to M2 than M1 macrophages [117]. In the later stages of tumor development, TAMs start to express markers that inhibit cytotoxic T cell activation, induce Tregs and support survival of tumor cells. Interaction of increased PD-1 expression on TAMs with PDL-1 ligands expressed on T cells leads causes suppression of the latter [118]. Additionally, TAMs secrete growth factors (e.g. EGF) which promote vascular development and enzymes (e.g. MMP9) that can digest the extracellular matrix leading to metastasis [119, 120].

Clinical studies indicate that the accumulation of TAMs within tumors is associated with poor outcome. TAMs have critical roles in the development and progression of cancer by inducing an immunosuppressive microenvironment. Thus, they may be both biomarkers of disease severity and potential targets for cancer therapy.

1.3.2.4 Effects of TLR Agonists on human monocytes and macrophages

Monocytes and macrophages constitute the first line of defense against pathogens. They express a wide range of PRRs. Human monocytes express TLRs 1,2,4,5,6,7 and 8 [121, 122]. TLR ligand stimulation can result in different pathway activation leading to a variety of monocyte and macrophages responses.

The TLR4 ligand LPS was the first to be identified as an M1 macrophage inducer. Recognition of LPS results in secretion of pro-inflammatory mediators. Repeated exposure to LPS can result in desensitization of macrophages as they start to over-express genes associated with pathogen recognition but only low amounts of pro-inflammatory cytokines. Desensitization of macrophages to LPS is induced by high levels of p50 subunit expression of NF- κ B which impairs STAT1 phosphorylation and type I IFN production [123].

TLR2 can be activated to a variety of stimuli including gram⁺ bacterial wall components, fungi, helminths, and synthetic compounds such as tri- and diacylated lipopeptides. TLR2 recognizes these components by interacting with other receptors such as TLR1, TLR6, CD14, CD36 and Dectin-1 [124-126]. Similar to most of the TLRs, downstream adaptor MyD88 is involved in the activation of MAPKs and NF- κ B [127]. TLR2^{-/-} mice are highly susceptible to infection, a finding that indicates the importance of TLR2 in protecting the host from bacterial infection [128, 129]. It was shown that TLR2 agonist stimulation can lead to generation of macrophages with suppressive functions. The TLR1/2 agonist PAM3CSK4 (PAM3) induced healthy human monocytes to differentiate into suppressive macrophages with strong endocytic ability. PAM3-generated macrophages also gained suppressive features such as inhibition of T cell proliferation [130, 131]. Stimulation of human monocytes with PAM3 down-regulated co-stimulatory molecule expression by secreting high levels of IL-10 [132]. Analysis of downstream pathway analysis during the differentiation of monocytes into macrophages revealed that NF- κ B and Akt were critical to general process. Whereas, p38 MAPK and PTGS2 were involved in the differentiation of pro-inflammatory or immunosuppressive macrophages and ERK & JNK contribute to the generation of M2 macrophages by PAM3 stimulation but not MCSF [131].

TLR7 and 8 are members of a genetically and structurally related subfamily of TLRs that recognize single-stranded RNA. TLR8 is expressed mainly on monocytes/macrophages whereas TLR7 is preferentially expressed on pDCs and B cells [133]. Activation of TLR8 on monocytes and mDCs leads to the production of TNF α , IL-12 and IL-10 [134]. By comparison, stimulation of TLR7 only induced type I IFN production by pDCs and not monocytes.

Combining TLR agonists with IFN γ leads to synergistic activation by enhancing TLR mediated NF- κ B activation. Combining IFN γ with TLR 7 or 8 agonists improved the tumoricidal activity, production of NO and pro-inflammatory cytokine production of the generated macrophages while decreasing IL-10 secretion [135, 136]. Using both IFN γ and TLR agonists results in strong M1 macrophage phenotype and thus can improve macrophage targeted cancer immunotherapy [135].

1.4 Autoimmune and inflammatory diseases

1.4.1 SLE

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder that typically affects women [137, 138]. It affects many organs including skin, joints, central nervous system and kidneys. The pathogenesis is linked to dysregulated innate and adaptive immune responses to nucleic acid containing cellular particles. The most characteristic clinical feature of SLE is the production of anti-nuclear autoantibodies (ANAs) [137]. ANA containing immune complexes deposit in many organs. The most severe clinical pathology is caused by the deposition of these complexes in the kidneys, which leads to the destruction of glomeruli, nephritis and proteinuria [139]. The course of SLE is variable among patients and usually presents itself in flares. The variability is characterized using criteria set by the American Rheumatism Association (ARA) presented in the table (Table 1.1). The presence of 4 disease features among the 11 listed qualifies a diagnosis of SLE [140].

Table 1.1: Diagnosis criteria of SLE by the American Rheumatism Association adapted from [142].

<u>Criteria</u>	<u>Prevalence in patients</u>
Malar Rash	27-63%
Discoid Rash	21%
Photosensitivity	38-60 %
Oral Ulcers	16%
Arthritis	42-95%
Serositis	25-45%
ANA blood test	99%
Renal disorder	22-50%
Neurologic disorder	2-25%
Hematologic alterations	81%
Immunological alterations	27-56%

The etiology of SLE remains unknown. However, like most of autoimmune diseases genetic and environmental factors are known to contribute to SLE. Deficiencies in complement pathway gene products which are involved in the clearance of cellular debris have an impact on SLE susceptibility [141]. Moreover, the extreme preponderance of lupus in women has been associated to some degree with expression of estrogen receptor 1 plus undefined immunomodulatory genes on the X chromosome [142]. Environmental factors also promote the development of SLE. Specifically, UV light, tobacco smoking and certain drugs (particularly antibiotics) have been associated with disease development [137].

An important step towards understanding and defining SLE as a disease of the immune system was taken by Dr. Hargraves in 1948, who identified the LE cell or LE body [143]. The LE cell is a neutrophil or macrophage in the bone marrow with distinctive morphology resulting from the phagocytosis of nuclear debris[143]. It was then discovered that the LE cell resulted from the uptake of ANA, since adding ANA to bone marrow cells resulted in the generation of the LE cell phenotype [144]. Currently, a number of factors are known to contribute to the pathogenesis of SLE, the most crucial ones can be listed as; (i) production of pathogenic autoantibodies, (ii) defects in cell death or debris clearance and (iii) lack of leukocyte regulation [142, 145].

Loss of self-tolerance and elevation of serum ANA levels represent a crucial first step in the development of SLE [146-148] while apoptotic cell debris are thought to be the major source of lupus autoantigens, in a normal healthy individual, apoptotic bodies are rapidly cleared. Defects in their clearance can lead to accumulation of apoptotic bodies in serum. In mice models of lupus high serum levels of nucleosomes are reported [149]. Moreover, injection of apoptotic bodies into non-autoimmune strains of mice such as C3H, BALB/c and C57BL/6 can elicit the development of serum autoantibodies similar to those observed in SLE [150]. These studies support the notion that apoptotic cell accumulation is responsible for antinuclear autoantibody formation. Studies also indicate that immune complexes activate pDCs through TLR signaling. IFN α secreted by activated pDCs can then directly stimulate B cells to produce antibodies [151].

Along with autoantibody production B cells contribute to SLE pathogenesis through other pathways. It was shown that B cells of SLE patients are more sensitive to antigens, cytokines and other stimuli [152]. They also have increased expression of surface molecules related to co-stimulation such as CD40L(CD154), CD80 and CD86 [140]. Increased levels of B cell activity factor (BAFF) was associated with the presence of autoantibodies [153]. BAFF increase survival of autoreactive B cells and lead to increased autoantibody production, [154]. Hence, administration of a soluble receptor for BAFF, TACI-Ig, was effective in treating murine lupus [155].

Studies in murine models of lupus also indicated that CD4⁺ T cells are important contributors in the production of autoantibodies [154]. Excess number of activated helper T cells induce the differentiation and activation of autoantibody producing B cells. Antigens are taken up by professional antigen presenting cells (APC) and presented to T cells. Activated T cells in turn stimulate B cells to produce pathogenic autoantibodies through their surface HLA molecules and other co-stimulatory molecule interactions such as CD40-CD40L, B7/CD28/CTLA-4 [141, 154].

Recent studies suggest that innate immune cells are also important contributors to disease pathogenesis in SLE. Abnormalities in monocyte and macrophage phenotype and function have been described and associated with SLE [156]. Monocytes/macrophages of SLE patients have a reduced capacity to phagocytose apoptotic materials [157]. Decrease in the clearance of apoptotic cells by macrophages is associated with decreased CD44 expression by monocytes [158]. Moreover, these cells contribute to disease pathogenesis through the secretion of cytokines and chemokines.

The conventional treatment approach for SLE involves a combination of immunosuppressive drugs, steroidal and non-steroidal anti-inflammatory agents and antimalarial drugs [159]. Belimumab, a fully humanized monoclonal antibody directed against B lymphocyte stimulator, is currently the only targeted treatment for SLE [160]. More B-cell targeted therapies and agents that target interferon or lymphocyte signaling are being evaluated in clinical trials

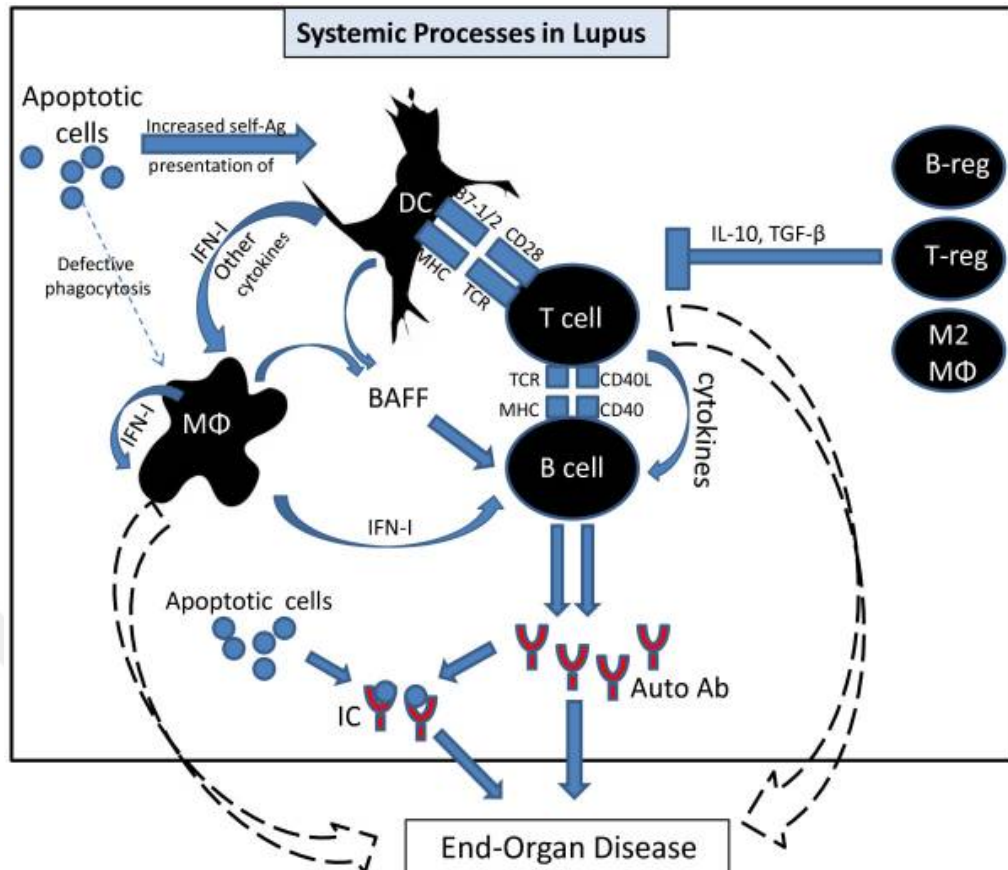


Figure 1.2 Summarized illustration of disease pathogenesis in SLE [154].

The availability of many murine models of lupus facilitates our understanding of disease pathogenesis and contributes greatly to the development of new therapies. There are a wide variety of lupus murine models including spontaneous, inducible and transgenic models [161]. Different murine models of the disease are useful to understand the etiology and each model shares attributes with lupus observed in humans and thus provides different aspects to study [162].

One of the first spontaneous lupus models described was using New Zealand Black crossed with New Zealand White mouse, the NZB x NZW F1 (NZB/W) [163]. NZB/W mice develop a disease closely resembling to human lupus. The disease is manifested by autoantibody production, glomerulonephritis and mild vasculitis [164]. Splenomegaly and hypergamma-globulinemia is also observed. However, these mice lack clinical other manifestations of human lupus such as rash or arthritis. Advanced disease typically appears at 6 months of age with a sex prevalence in females just like in humans. At 9 months of age, mortality approaches 50%. NZB/W mice have been used in the preclinical testing of many therapies that were

taken to clinical trial [165, 166]. A disadvantage of this model is that disease develops over a prolonged period of time. Thus, ways to accelerate disease progression have been investigated by administering adenoviruses that express interferon or by injecting TLR7 or 9 agonists [167, 168]. Over 20 different inbred strains related to NZB/W were generated, and are called NZM strains [169]. NZM2410 and NZM2328 are the two strains that are being used in the lupus research today. These strains help to define the genetics of lupus by backcrossing them to normal C57BL6 mice [170]. The advantage of the NZM strains is that they can be bred to each other unlike NZB/W. Similar to the NZB/W they develop glomerulonephritis and autoantibodies but not vasculitis [171]. A third spontaneous lupus strain is the MRL/lpr mice which was generated by intercrossing four different strains (LG, B6, AKR and C3H) [172, 173]. MRL/lpr mice have a single gene mutation in the lpr gene that encodes the Fas receptor [174]. They develop lupus like manifestations including autoantibodies, arthritis, cerebritis, skin rash and vasculitis [175]. Disease is accelerated in females but not as prominently as in NZB/W mice. MRL/lpr mice show a 50% mortality in 6 months. Even though MRL/lpr strain is used in the assessment of candidate treatment for lupus due to its early onset, it is not a perfect model for human lupus because disease is not primarily driven by IFN α but more IFN γ . Additionally, the lpr mutation that leads to accelerated disease is not present in human [176].

The most widely used inducible model of lupus is the pristane model [177]. Pristane, a mineral oil, is injected intraperitoneally to BALB/C mice to induce peritoneal irritation. It was reported by Satoh et al that after a number of months pristane injection leads to a lupus like disease in mice manifested by glomerulonephritis, mild arthritis and autoantibodies [178]. Inducible models of lupus help provide knowledge about the environmental factors that contribute the pathogenesis of disease.

Transgenic models of lupus allowed the investigators to understand the role of specific gene products or proteins contributing to disease. One of the earlier transgenic model introduced immunoglobulin heavy and light chain genes that code for autoantibodies [179, 180]. Using transgenic models, insight was gained into how tolerance was maintained. Moreover, over-expression of immune modulatory factor

genes provided more knowledge of their role in tolerance [181]. Gene knockout models have also been extensively studied to understand pathogenetic mechanisms. An example is when TLR7 and TLR9 knockouts were bred onto the MRL/lpr background. It was reported that TLR7 deficient mice developed less severe disease whereas absence of TLR9 led to accelerated disease [182-184].

1.4.2 Vasculitis

Vasculitis is an inflammation of blood vessels, which leads to vessel wall thickening and eventually to end-organ ischemia. It can also lead to the formation of aneurysms and/or breaches in vessel integrity resulting in hemorrhage [185]. Fundamentally, vasculitis is a primary inflammatory process whose underlying mechanism remain unidentified [186]. However, vasculitis can arise as a secondary process in other diseases including infection, malignancy and rheumatic diseases. Primary and secondary vasculitis differ in the age of onset, gender, and other clinical manifestations including symptoms, organ involvement and in the treatment approach [185].

Vasculitis is typically classified into three subsets based on the size of the affected vessels: (i) small-vessel, (ii) medium-vessel and (iii) large-vessel [186, 187]. This classification was adopted by the International Consensus Conference on Nomenclature of Systemic Vasculitides, Chapel Hill in 1994 [188].

Small vessel vasculitis involves the smallest arteries, arterioles, capillaries and venules [186]. It can coexist with medium-vessel vasculitis. The determining factor for small vessel is the behavior of disease. It can occur systemically or locally. Histopathology, immunohistology and autoimmune serology are important in the determination. Small vessel vasculitis is classified into three based on immunopathological differences; (i) antibody mediated, (ii) immune complex mediated and (iii) 'Pauci immune' or anti-myeloperoxidase perinuclear ANCA [186].

Medium vessel vasculitis affects small muscular arteries such as temporal artery, arcuate artery and branches. It can occur both systemically and locally. Systemic medium vessel vasculitis can result in granuloma formation [186].

Assessment of small and medium vessel vasculitis is done based on the Birmingham Vasculitis Activity Score (BVAS) scoring tool, which includes 66 clinical features from nine organ systems. Each feature is scored based on its clinical relevance. The list includes most of the features observed in patients with different forms of vasculitis, but patients do not show all the abnormalities at once [189, 190].

Large vessel vasculitis is observed in two main forms; Takayasu's arteritis (TAK) and giant cell arteritis (GCA). Similar histologic abnormalities are observed in both; however, the age of onset and targeted vascular structures are different [191, 192].

TAK usually occurs in the aorta whereas GCA lesions are localized in more peripheral medium-sized arteries affecting the branches of aorta [192-194]. TAK was first reported by a Japanese ophthalmologist Dr. Mikito Takaysu in 1908 [195]. It was defined by chronic granulomatous inflammation of the aorta and its branches. It is usually observed in young white women [189].

GCA is the most frequently observed form of large vessel vasculitis. It is observed more in northern Europe and two thirds of the affected are women over the age of 50 years [192, 196]. Dysregulation of the vessel wall and the immune system in GCA is driven by 3 main cell types; dendritic cells (DC), CD4⁺ T cells and macrophages [192, 193, 197, 198]. The first step is the activation of DCs which are found in the vessel walls. DCs are activated by danger signals through their TLRs. The source of these danger signals could arise from a variety of sources including infectious agents, products of tissue breakdown, metabolic abnormalities, and deposition of irritating agents [199-201]. Activated DCs then induce the recruitment of CD4⁺ T cells, monocytes and macrophages to the vessel wall where they penetrate into tissue and form granulomas [193, 202, 203]. Monocytes mature into macrophages in the lesion site based on the environmental factors. Activated macrophages and DCs fuse together to form giant multinucleated cells, a hallmark of GCA [204]. It was reported that macrophages in the granulomas can produce pro-inflammatory cytokines such as IL-1, IL-6 and TNF α indicating that they display characteristic features of M1 macrophages (Figure1.3). IL-12 is essential for biasing T cells toward the Th1 lineage while IL-6, TGF- β and IL-23 provide signals for the differentiation of Th17 cells. M1 macrophages are found frequently in

granulomatous infiltrates. It has also been proposed that M2 macrophages are present in granulomas as a counter mechanism to support tissue repair [205, 206].

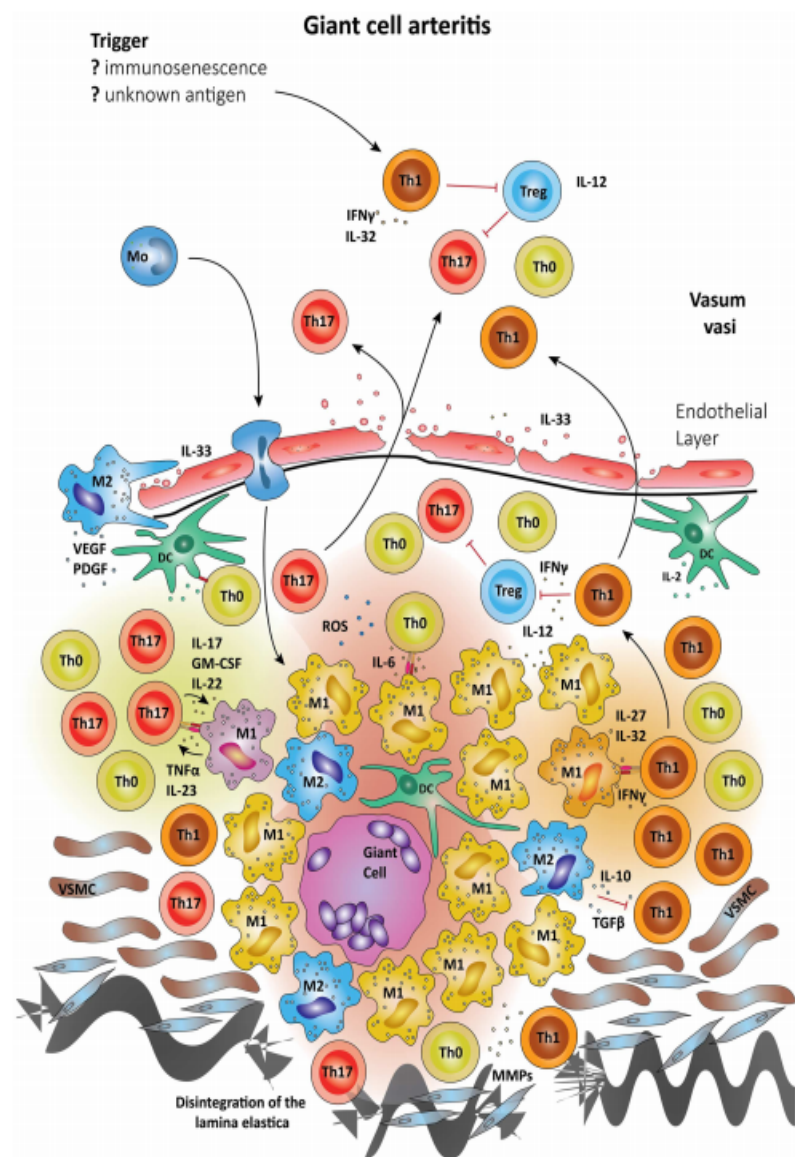


Figure 1.3 Schematic summary of the cellular pathways involved in the formation of granulomatous lesions in GCA[204].

In general patients with GCA receive glucocorticoids or immunosuppressive drugs. [207]. Similar to SLE, inhibitors that target lymphocyte activation such as T cell modulators, IL-6 receptor antagonists and more immunosuppressive drugs are being evaluated in clinical trials [208].

1.4.3 Myositis

The idiopathic inflammatory myopathies (IIMs) also known as myositis, are a group of autoimmune disease affecting muscles with significant morbidity and mortality if not treated properly [209, 210]. Classical clinical manifestation of all IIMs are muscle weakness and chronic inflammation in skeletal muscles which further affects other organs such as skin, joints, lungs, gastrointestinal tract and heart [210]. IIMs are classically classified into three main subsets based on the differences in clinical and histopathological features; (i) sporadic inclusion-body myositis (sIBM), (ii) polymyositis (PM) and (iii) dermatomyositis (DM) [211-213]. Classification of myopathies was initially done by the criteria created by Bohan and Peter in 1977, further research has demonstrated increased sub classifications was beneficial to understand and treat myopathies [211]. Thus, two additional forms have been described; nonspecific myositis and immune-mediated necrotizing myopathy (IMNM) [214]. It should be noted that there is no consensus on the classification of IIMs. Subsets based on the traditional classification will be defined in this section. IBM is more frequently observed in men than in women over the age of 50, PM is seen mostly in adults and DM can affect both children and adults [212]. Juvenile dermatomyositis is the most common type of myopathies that is observed in children. Even though the presentation of DM in both adults and juveniles are similar, it is suggested that the underlying mechanisms might differ due to the fact that adults suffer from more severe disease that can lead to cancer whereas it is milder in the juvenile patients [215]

The mechanism in the development of IBM includes a complex network of inflammatory and degenerative mechanisms. Muscle fibers are attacked by autoreactive T cells and antibodies. Moreover, accumulation of β -amyloid, tau and α -synuclein proteins within the muscle fibers are contributing to the development of the diseases. It was recently shown that production of nitric oxide and increased autophagic activities are contributing to muscle fiber damage [212, 216].

PM and DM are characterized by low endurance and weakness of muscles. Clinically PM and DM are very similar, however DM also affects the skin along with muscles [217]. T cells, macrophages, dendritic cells and B cells are present in

the muscle biopsies of both DM and PM patients. Autoantibody production is also very common among patients. The presence of autoantibodies and immune cells in muscle biopsies indicate that immune mechanisms are involved in disease pathogenesis [218-220]. High levels of inflammatory cytokines such as IFN γ , IL-6, IL-1 β and TNF α and chemokines that attract immune cells are reported in PM patient muscles [221]. Recent studies indicate that stimulation of TLRs by endogenous antigens might be involved in the inflammatory processes [222].

Treatment options for myositis include steroids, antimalarial medications, intravenous immunoglobulins and conventional immunosuppressive drugs. The role of antibodies used in different disease conditions are also being considered as treatment approaches. Currently there is a lack of controlled clinical trials for the treatment of myositis due to the heterogeneity and rarity of the disease [223].

1.4.4 Macrophages in autoimmune and inflammatory diseases

As previously mentioned, an alteration in the balance between M1/M2 macrophages is found in many disease states. For example, in cancer a reduction in the M1:M2 ratio leads to a decrease in tumoricidal M1 macrophages and increase in immunosuppressive M2 macrophages (that interfere with the tumoricidal activity of CTL). In chronic inflammatory conditions, the production of reactive oxygen species by M1 macrophages can increase the likelihood of cancer developing. In this context, patients with Crohn's disease and other inflammatory bowel conditions have an increased incidence of colorectal cancer [224, 225]. By comparison, an increased M1:M2 ratio is associated with the development of several autoimmune diseases [111]

1.4.4.1 Macrophages in SLE

Recent studies indicate that monocytes and macrophages contribute to the pathogenesis of SLE. An imbalance in the M1:M2 macrophage ratio is found in lupus flares while the frequency of inflammatory monocytes is increased in SLE patients [226, 227]. Abnormalities in monocyte/macrophage surface marker expression and functions have also been described in SLE.

Monocytes express a variety of surface markers that regulate their response to environmental factors. Abnormal expression of these markers can contribute to the inflammatory milieu. Receptors involved in phagocytosis and secretion of inflammatory cytokines including Fc gamma receptors (FcγRs) were among these dysregulated surface molecules [228]- [229].

Overexpression of co-stimulatory and adhesion molecules on monocytes can lead to aberrant migration of monocytes and lymphocyte activation. Expression of the CD40 co-stimulatory molecule is reported to be increased on lupus monocytes when compared to healthy controls [232]. Interaction of CD40 with its ligand CD40L induces lymphocyte activation, proliferation and initiation of immunoglobulin isotype switch [233]. Hence abnormal expression of CD40 on monocytes might facilitate the proliferation of autoreactive lymphocytes and autoantibody production. Although the mechanism underlying the phenotypic changes on monocytes is unknown, genetic and environmental factors might be contributing [234, 235]. The serum cytokine milieu in SLE may also play a role in the increased expression of these surface molecules. Several groups have described that type I Interferon stimulated genes (ISGs) are highly expressed in PBMC of lupus patients [227, 236, 237]. Elevated levels of type I IFN in lupus patients can also contribute to the inflammatory phenotype of monocytes. Moreover, activation of IRF/STAT signaling pathway by type I IFNs skew macrophages toward an M1 phenotype [238].

Secretion of pro-inflammatory cytokines by monocytes and macrophages also promote activation of autoreactive T and B cells [156]. Activation of TLRs through endogenous nucleic acids induces maturation of monocytes and production of inflammatory cytokines such as TNFα [239].

As mentioned before, SLE patient macrophages show a defect in their ability to remove apoptotic cells that supports the production of autoantibodies [157]. This deficiency is linked to both intrinsic defects in the macrophages and an increased frequency of M1 macrophages [156]. Expression of CD44, a surface marker associated with the clearance of apoptotic cells, is decreased in lupus patients [158]. On the other hand, increased levels of pro-inflammatory cytokines including IL-12,

TNF α , IFN γ and type I IFNs are likely to be contributing to increased frequencies of M1 macrophages in lupus. These cytokines were shown to support the generation of M1 macrophages through pathways including JAK/STAT1, NF κ B and interferon regulatory factors [236, 238, 240]. Moreover, chemokines that promote monocyte infiltration and macrophage differentiation/proliferation were reported to be elevated in mice and human lupus patients and may be contributing to this process [241, 242].

Studies involving the in vivo depletion of macrophages emphasized the contribution of these cells to murine lupus [242]. Genetic deletion of colony stimulating factor (CSF-1), its receptor CSF-1R or depletion by an inhibitor in MRL/lpr mice resulted in decreased infiltration of kidneys and less glomerulonephritis [241, 243]. Further studies showing reduced disease severity in both rat and mouse upon depletion of macrophages or inhibition of their recruitment to kidneys support the conclusion that macrophages are important contributors to disease pathogenesis [244-250]. Unfortunately, depleting macrophages can have serious side effects as the susceptibility of the host to bacterial infections increases greatly. Another study where adoptive transfer of in vitro differentiated M2 macrophages reduced disease severity in lupus prone mice showed the beneficial effects of M2 macrophages in slowing disease progression, an approach that did not require depletion of macrophages [251]. All of these studies raise the possibility that macrophages are a valuable therapeutic target in the treatment of SLE.

1.4.4.2 Macrophages in Vasculitis and Myositis

In vasculitis, pathology is initiated by the infiltration of monocytes/macrophages and T cells into the vessel wall. Monocytes counts were elevated in GCA patient blood and increased numbers of inflammatory monocytes were subsequently observed within the infiltrates of the adventitia, media and intima of the vessel wall. [252]. The monocytes and macrophages that migrated into vessel walls can produce inflammatory cytokines (a classical feature of M1 macrophages) and matrix metalloproteases that lead to vascular damage [252, 253]. IFN γ , a potent activator of M1 macrophages, was found abundantly in GCA involved arteries, and is believed to play a role in driving the granulomatous reaction and transformation of macrophages to giant cells in these lesions [254]. A study has shown that increased IFN γ

expression was accompanied by increased M1 macrophages in arteries suggesting that the inflammation was caused by Th1-mediated macrophage activation [255]. Reactive oxygen species secreted by activated macrophages also contribute to vessel wall injury [256]. It was proposed that the reactive oxygen species and hydrolytic enzymes produced by macrophages might be responsible for pathological changes in GCA because macrophages expressing iNOS and collagenase were found to accumulate in the intimal and interior medial layer of the vessels [257].

Unfortunately, there are not many studies available on the contribution of monocytes/macrophages to disease pathogenesis in myositis. However, muscle biopsies of DM and PM patient's show that macrophages are found along with T cells. These macrophages display different activation patterns and distribution in DM versus PM patients. These macrophages are capable of producing inflammatory molecules such as iNOS and TGF- β , consistent with their being M1 macrophages [258, 259]. All of the above findings indicate that macrophages can play a role in the pathogenesis of multiple inflammatory and autoimmune diseases.

1.5 Aims of the thesis

Macrophages play an important role in the initiation of immune responses and the maintenance of immune homeostasis. Alterations in their phenotype, function and activation state have been implicated in the pathogenesis of autoimmune and inflammatory diseases. Thus, regulation of their differentiation represents a novel strategy in the treatment of such conditions.

The first aim of this thesis was to investigate the ability of PAM3 to induce M2 macrophage differentiation from healthy and patient monocytes. Blood samples from different autoimmune conditions were processed and macrophages were generated from sorted monocytes using various ligands. Expression of cell surface molecules was analyzed to classify macrophages and understand their behavior. I analyzed their functions by measuring endocytic ability and secretion of both pro- and anti-inflammatory cytokines.

Based on findings from human samples, evaluating the therapeutic potential of PAM3 was evaluated as the second aim of this thesis. I used the widely accepted model of spontaneous murine lupus: NZB/W F1 mice. PAM3 was administrated intraperitoneally to these animals at a dose and frequency previously found to be effective in the treatment of allergic asthma and cardiac dysfunction [260-262]. Disease progression was monitored weekly by examining proteinuria as an indicator of kidney damage. I then evaluated the phenotype and function of macrophages from PAM3 treated mice when they reached 6 months of age. Further analysis was done using immunohistochemistry and ELISA to investigate kidney damage and finally survival was monitored in a separate cohort of mice.

The third part of this thesis focused on the immunomodulatory effects of CpG ODNs encapsulated in different types of liposomes. I investigated the effect of each liposome combination on human PBMC and murine splenocytes. After establishing enhanced immunostimulatory effects in both human and mice, CpG ODN combinations encapsulated into liposomes was tested as vaccine adjuvants in an in vivo tumor model using the OVA expressing B16 melanoma cell line. The effect of the formulation as a vaccine adjuvant was evaluated by measuring anti-OVA responses after primary and secondary injections and tumor sizes were monitored.

Chapter 2

Materials & Methods

2.1 Materials

2.1.1 Cell Culture Media and Standard Solution Components

DMEM high glucose without L-glutamine (Catalog # 12-614F), FCS (Catalog # 14-502F), 1X PBS (Catalog # 17-516F), 10X PBS (Catalog # 17-516Q), RPMI-1640 without L-glutamine (Catalog # 12-167F), RPMI-1640 without phenol red & L-glutamine (Catalog # 12-918F) were purchased from Lonza, USA. Sodium Pyruvate (100 mM; Catalog # 11360-070), L-glutamine (200mM; Catalog # 25030-081) and Pen Strep (5,000 units/ml Penicillin and 5,000 µg/ml Streptomycin; Catalog # 25070-063) were supplied from ThermoFisher Scientific, USA; BSA (Catalog # BSA-50) from Rockland Immunochemicals, USA, and Tween-20 (Catalog # P1379) from Sigma-Aldrich, USA. Complete RPMI-1640 and DMEM were prepared by adding 1 x Sodium Pyruvate and 1 x L-glutamine and different amounts of FCS (heat-inactivated at 56° C for 1 hour). Oligo RPMI-1640 was prepared using FCS heat-inactivated at 65° C and also contained 1 x Pen Strep. For each experiment FCS percentage of the media is specified in Methods Section.

2.1.2 TLR Ligands

Receptor specificities, catalog numbers, vendors and standard working concentrations of TLR ligands used in stimulation experiments are listed in Table 2.1.

Table 2.1: TLR Ligands

Name	Specificity	Catalog #	Company	Working Concentration
Pam3CSK4	TLR1/2	tlrl-pms	Invivogen (USA)	1 µg/ml
R848	TLR7/8	tlrl-r848-5	Invivogen (USA)	1 µg/ml

2.1.3 Oligodeoxynucleotides

D and K- type CpG ODNs were bought from Alpha DNA (Montreal, Canada).

Table 2.2: Sequences of ODN used.

Upper case letters: Phosphorothioate linkage between bases, lower case letters:

Phosphorodiester linkage between bases

<u>K-type CpG ODN</u>		
1555	15mer	5'-GCTAGACGTTAGCGT-3'
1555-3CG	20mer	5'-GACGTTGACGTTGACGTTGG-3'
<u>D-type CpG ODN</u>		
D35	20mer	5'-GGTgcatcgatgcaggggGG-3'
D35-3CG	20mer	5'-GGTcgcgatcgatcgaggggGG-3'

2.1.4 Recombinant Cytokines

Human recombinant cytokines were used at a concentration of 50-500 ng/ml. All the reagents shown in Table 2.3 were purchased from Miltenyi Biotec (Germany).

Table 2.3: Recombinant Cytokines

Name	Catalog#
Human IFN γ , research grade	130-096-873
Human M-CSF, premium grade	130-096-492

2.1.5 Flow Cytometry Antibodies

Fluorescence-conjugated antibodies used for the flow cytometric analysis of the cells are listed in Table 2.3. Dilutions were made in staining buffer (2%BSA in PBS).

Table 2.4: Flow Cytometry Antibodies

Name	Clone	Catalog #	Company	Dilution
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Name	Clone	Catalog #	Company	Dilution
Anti-human Mature Macrophage	eBio25F9 (25F9)	14-0115-82	Thermo Fisher, USA	1:25
Anti-human CD14	MΦP9	560349	BD Pharmingen, USA	1:25
Anti-human CD16	B73.1	369716	Biolegend, USA	1:25
Anti-human CD163	GHI/61	333618	Biolegend, USA	1:25
Anti-human CD40	HB14	313014	Biolegend, USA	1:25
Anti-human CD44	BJ18	338810	Biolegend, USA	1:25
Anti-human 273	24F.10C12	329606	Biolegend, USA	1:25
Anti-human IL12	C11.5	559329	BD	1:5
Anti-mouse CD45	30-F11	103106	Biolegend, USA	1:100
Anti-mouse CD3	145-2C11	100312	Biolegend, USA	1:100
Anti-mouse CD19	6D5	115512	Biolegend, USA	1:100
Anti-mouse CD11b	ICRF44	301324	Biolegend, USA	1:100
Anti-mouse F4/80	BM8	123116	Biolegend, USA	1:100
Anti-mouse CD11c	N418	117310	Biolegend, USA	1:100
Anti-mouse CD206	C068C2	141704	Biolegend, USA	1:100

2.1.6 ELISA Antibodies

Immulon 2HB microtiter plates purchased from ThermoFisher Scientific (USA). 1 x PBS supplemented with 0.05% Tween 20 used as washing buffer. All recombinant proteins were purchased from R & D (USA). Clone, Catalog number and dilutions are indicated in Table 2.4. Phosphatase-streptavidin was purchased from BD Biosciences (USA) and used 1:5000 diluted in blocking buffer (2% BSA in PBS). PNPP substrate was purchased from Southern Biotech (USA). Spectra Max M5 Microplate Reader, and SoftMax Pro Acquisition and Analysis Software (both Molecular Devices, USA) were used for data acquisition.

2.1.7 Lipids

Cholesterol was purchased from Sigma Aldrich (USA). L-α-Phosphatidylcholine (PC), 3α-[N-(N',N'-Dimethylaminoethane)-carbamoyl] Cholesterol Hydrochloride

(DC-Chol), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE),) were all purchased from Avanti Polar Lipids (Alabama,USA).

2.2 Methods

2.2.1 Patients and Controls

2.2.1.1 Isolation of PBMC from human blood

Blood was obtained from SLE patients and healthy volunteers after obtaining IRB-approved written consent (NIDDK/NIAMS IRB, Bethesda, MD). Blood samples were diluted with 1x PBS and slowly layered over Ficoll (Histopaque 1077, sigma Aldrich, USA) at a volume:volume ratio of 2:1. Blood was then centrifuged at 400 g for 30 min without deceleration. Cloudy interphase was collected and transferred to a 50ml falcon tube using a sterile Pasteur pipette. Samples were then washed twice with 50ml RPMI-1640 supplemented with 2 %FBS and centrifuged at 400g for 10 min. PBMCs were then counted with KX-21N Automated Hematology Analyzer (Sysmex, USA).

2.2.1.2 Sorting of Monocytes

Monocytes were isolated from PBMC using MACS human CD14 microbeads according to the manufacturers' protocol. Cells were first labeled with magnetic beads by incubating 10^7 cells with 20 μ l CD14 microbeads for 15min at dark. Meanwhile LS columns were prepared for magnetic separation by passing 3 ml of MACS buffer. After 15 min incubation in the dark at 4° C cells were washed, resuspended in 500 μ l of MACS buffer and loaded onto magnetized LS columns in 500 μ l of MACS buffer. Columns were washed 2 times with 3 ml of MACS buffer. CD14⁺ cells collected by removing the magnet and flushing with 5 ml of MACS buffer. Monocytes were counted with KX-21N Automated Hematology Analyzer (Sysmex, USA). 10^5 cells were separated for the analysis of day 0 monocytes with flow cytometry.

2.2.2 Stimulation of monocytes and phenotype analysis

10⁵ purified monocytes/well were seeded into 96-well flat bottom plates (Corning) in 200 µl RMP-1640 supplemented with 2% FBS. Cells were stimulated for 5 days at 37°C with 1 µg/ml PAM3CSK4 (PAM3) (Invivogen), 250 ng/ml MCSF (Miltenyi Biotec), 250 ng/ml IFN γ (Miltenyi Biotec) or 1 µl /ml R848 (Invivogen). Previous studies established that these were the optimally stimulatory concentrations of each agent ([14,15]). Cells were maintained in medium consisting of RPMI 1640 (Lonza) supplemented with 2% heat-inactivated fetal calf serum 25 nmol/L HEPES, 1 mmol/L sodium pyruvate, non-essential amino acids (NEAAs) and 0.0035% 2-mercaptoethanol (2-ME).

Supernatants were removed, and cell pellets were resuspended in 2% BSA PBS. Human cells were stained with 2 µl of fluorochrome-conjugated Abs specific for human CD14, CD16, 25F9 (eBioscience), CD163, CD40, CD44 and/or CD273 (Biolegend) for 20 min in 50 µl of PBS – 2% BSA on ice. Samples were then washed and fixed using 50 µl of Fixation Medium A (Fix & Perm Cell Fixation & cell permeabilization Kit, ThermoFisher Scientific, USA). Cells were incubated at room temperature, dark for 15 min. Differentiation of monocytes into macrophages was determined by the acquired 25F9 expression. M2 macrophages were characterized by the expression of scavenger receptor CD163, which is only present on M2-like macrophages but not M1-like macrophages.

2.2.3 Functional analysis of differentiated macrophages

2.2.3.1 Endocytosis assay

Human macrophages generated after 5 days of stimulation were incubated with 25 µg/ml of pH-Rodo Zymosan (Invitrogen) particles or medium (as background control) for 45 min at 37° C. Cells were then washed and stained with 25F9 and CD163. The uptake was calculated by subtracting the background MFI values (media incubated) from pH-Rodo Zymosan MFI levels determined by LSR-Fortessa.

2.2.3.2 Cytokine detection by ELISA

Immuol 2HB-microtiter plates were coated with human anti-TNF α (R&D Systems), mouse anti-IL12 (BD Biosciences), then blocked with 2% BSA-PBS for 2 hr. After washing, culture supernatants or serially diluted sera were added overnight at 4° C. Plates were washed, incubated with biotin-labeled secondary Ab and developed by adding phosphatase-conjugated streptavidin (AKP) followed by p-nitrophenyl phosphatase (pNPP) substrate (Southern Biotech). Optical density was measured using a SpectraMax M5 microplate Reader and SoftMax Pro Acquisition and Analysis software (Molecular Devices).

2.2.3.3 Staining for intracellular cytokines

For the detection of intracellular IL-12 PBMC were stimulated with MCSF, PAM3, IFN γ or R848 for 24 hr along with a 1:1,000 dilution of GolgiPlug (BD Biosciences). Cells were stained and fixed as previously described. Fixed cells were resuspended in 50 μ l Permeabilization Buffer B containing 2.5 μ l fluorochrome-conjugated IL-12 or isotype control. The mixture was incubated for 20min at dark, room temperature and washed again as described.

2.2.4 Liposome Preparation

Phospholipid and cholesterol stock solutions were adjusted to 10mg/ml in chloroform and stored at -20 C. Based on the ratios indicated in the table... lipids were mixed. Chloroform was then evaporated with rotary evaporator until a thin film was formed in the round bottom flask. The film was passed through nitrogen gas to discard the remaining oxygen to prevent peroxidation of the lipids. Large multilamellar vesicles were obtained from the film by shaking the flasks rotationally with 1ml 1X PBS and glass beads. For the generation of small unilamellar vesicles, liposomes were sonicated 10-15min for 30s durations with an amplitude of 50% (Vibracell SONICS, USA).

For the encapsulation of CpG ODNs, 150 mg CpG was added to 4.5 μ mol of liposomes. The mixture was snap frozen in liquid nitrogen and lyophilized overnight (Benchtop K, Virtis, USA). Encapsulation of CpGs into liposomes was achieved by the controlled dehydration methods. 20 μ l of DNase RNase free water was added to

the lipid/CpG powder and vortexed for 15s every 5 min for 25 min. Another 20 μ l of PBS was then added and incubated for 10 min at room temperature. Finally, to have a CpG concentration of 1 μ g/ μ l, 110 μ l of PBS was added and liposomal CpGs were stored at 4 °C until further use.

Table 2.5: Lipid ratios for liposome preparation

Liposome	Lipid composition (molar ratio)
Neutral	PC:Chol (1:1)
Cationic	DC-Chol:PC:DOPE (4:6:0.06)

2.2.5 Stimulation of human PBMC and splenocytes

PBMC were isolated by Ficoll density centrifugation from healthy donor blood samples that were collected into vacutainer tubes containing EDTA as anti-coagulant (BD Vacutainer, purple cap). Recovered PBMCs were seeded at 1×10^6 cell/ml in 250 μ l in 96-well plates and stimulated with free or liposomal CpG ODNs.

Single cell suspensions were prepared from spleen of C57BL6 mice and 2.5×10^5 cell/well seeded onto 96-well flat bottom plates and stimulated.

2.2.6 Confocal Microscopy

RAW cells were seeded onto 35mm glass bottom dishes (MatTek, USA) and incubated for 1 hr to let them attach. Afterwards, cells were stimulated with Cy5 or Cy3 labeled liposomal or free CpG ODNs for 3 hr. Cells were then incubated 30 min with LysoTracker-FITC (1:50) to label late endosome followed by 2 washing steps and imaged live in glass bottom dishes. For early endosome cells were incubated with Transferrin-FITC (100 mg/ml), for 30 min followed by 2 washing steps. Cells were then fixed and mounted on the microscopy slides for imaging. Images were taken at 63X with a LSM 710 microscope.

2.5.7 Internalization Studies

Human PBMC were stimulated with FITC CpG ODNs, for 1 hr and 6 hr at 37°C to assess binding and internalization and at 4°C for binding only. Cells were labeled with BDCA-2-PerCpCy5.5 and CD123-APC to gate pDCs. Samples were then

analyzed by BD Fortessa, and to calculate internalization, values at 4°C (binding) were subtracted from values at 37°C (binding and internalization).

2.2.8 In vivo experiments

2.2.8.1 Tumor experiment

Six to eight-week-old C57BL/6 mice were immunized intraperitoneally on days 0 and 15 with OVA (1 µg/mice) or OVA encapsulated liposomes (0.5 µmol/mice) along with CpG ODNs (5 µg/mice). Sera were collected on day 29 and antibody levels were determined by ELISA to monitor OVA-specific antibody responses. Immulon 1b plates were coated overnight with 50 µl of OVA (10 µg/ml) at 4°C and blocked with PBS-5% BSA. Mouse sera was serially diluted four-fold eight times and OVA specific antibodies were detected using goat anti-mouse IgG1 or IgG2c alkaline phosphatase conjugate (1:2000) followed by PnPP substrate addition. Color development was followed at OD 405 nm using microplate reader. Eleven days after the booster injection (day 49) mice were challenged with subcutaneous injection of B16-OVA (1x10⁶ /mouse) melanoma cells. After tumors reached a palpable size, tumor volumes were monitored using a caliper every other day and recorded as length x width x height.

2.2.8.2 Evaluation of PAM3's effect on lupus prone mice

Female NZB and male NZW mice purchased from The Jackson Laboratory (Bar Harbour, ME) were bred. Female NZB/W F1 offspring were housed under pathogen-free conditions in the NCI animal facility. All experiments were reviewed and approved by the NCI Animal Care and Use Committee. Normal BALB/c and lupus-prone NZB/W mice were injected weekly with 100 µg of PAM3 (Invivogen) i.p. starting early (7 wk of age) or late (13 wk). Mice were sacrificed only when deemed in extremis by veterinary staff

2.2.9 Collection of peritoneal cavity cells

An incision was made on the abdominal skin of mice until the peritoneal membrane was exposed. Peritoneal cells were collected by injecting 5 ml of 3% FBS supplemented PBS with a 20 Gauge needle to peritoneal cavity. This was repeated

until around 15 ml of cell containing solution was collected. Cells were then centrifuged at 400g for 5 min and counted, the supernatant was discarded, and pellet was dissolved in 5% FBS supplemented RPMI media at the desired concentration.

2.2.9.1 Phenotypic analysis of macrophages by flow

Murine peritoneal cells were first incubated with Fc Block (Biolegend) for 15 min in 2% BSA PBS at room temperature. Cells were then washed and stained with fluorochrome conjugated Abs specific for murine CD45, CD11b, F480 and CD206 under the same conditions as described above. Stained cells were washed and fixed with Fix & Perm Medium A (Invitrogen).

2.2.10 Preparation of single cell suspension from mice spleens

Mice were sacrificed by cervical dislocation, their spleens were removed and transferred into 6 cm petri dish with pre-warmed 2% FBS supplemented complete RPMI media. Single cell suspensions were prepared by mashing the spleens on top of a 70 μ m strainer (Falcon) using the plunger of a 1 ml syringe. Strained was washed passing 10-20 ml media through and cells were centrifuged 5 min at 400g. Cells were washed and strained one more time and then counted with KX-21N Automated Hematology Analyzer. Cell concentration was set to desired concentration by the addition of 5% Oligo FBS supplemented RPMI media.

2.2.11 Blood sampling and serum collection from the mice

Blood was collected into 1.5 ml microtubes from the submandibular vein of mice using a 4-5mm Lancet. Tubes were incubated at 37° C for 20 minutes for blood to clott, then centrifuged at 13500 rpm for 10min. Serum was then collected into round bottom plates and stored at -20° C for further use.

2.2.12 Cytokine and Autoantibody ELISA from serum and splenocyte cultures

Immunol 2HB-microtiter plates were coated with mouse anti-TNF α (R&D Systems) or mouse anti-IL-10 (R&D Systems) and then blocked with 2% BSA-PBS for 2 hr. After washing, culture supernatants or serially diluted sera were added overnight at 4° C. Plates were washed, incubated with biotin-labeled secondary Ab and

developed by adding phosphatase-conjugated streptavidin (AKP) followed by p-nitrophenyl phosphatase (pNPP) substrate (Southern Biotech). Optical density was measured using a SpectraMax M5 microplate Reader and SoftMax Pro Acquisition and Analysis software (Molecular Devices).

IgG anti-dsDNA was detected by coating Immunol 2HB-microtiter plates with 4 µg/ml of CT-DNA (Sigma) in DNA binding solution (Abcam) for 4 hr. The plates were blocked with 2% BSA-PBS and incubated with diluted mouse serum as above. Bound Ab was detected with biotin labeled IgG (Invitrogen) followed by phosphate-conjugated streptavidin and pNPP substrate.

2.2.13 Proteinuria, BUN and Creatinine Measurements

The protein content of early morning urine was monitored using Albustix test strips (Siemens Heathineers). A couple of drops of urine was added to the strips and the proteinuria grade was monitored based on the colorimetric change.

BUN and creatinine levels were measured by colorimetric ELISA kits from Bioassay systems and Thermofischer respectively, from the serum of mice that were 6 mos of age.

2.2.14 Collection of Kidney and Staining

Kidney damage was evaluated in 2-3 µm formalin fixed paraffin embedded sections using Periodic Acid Schiff (PAS) and Masson trichrome staining. Pathological changes evaluated included glomerular activity (i.e., glomerular proliferation, karyorrhexis /fibrinoid necrosis, cellular crescents and hyaline deposits), tubulointerstitial activity (i.e., tubular dilatation and interstitial inflammation) and chronicity of the lesions (i.e., glomerulosclerosis, tubular atrophy, interstitial fibrosis) as previously reported [16]. Glomerular activity was evaluated on 30 glomeruli /sample and scored using 0-3 scale in which 0= no lesions, 1=lesions in <25% of glomeruli, 2=lesions in 25-50% of glomeruli and 3= lesions in >50% of glomeruli. Tubular activity and chronicity indices were based on a 0-4 scale as 0=no lesions, 1=lesions in 1-10%, 2=11-25%, 3=>25-50% and 4=>50%. Glomerulonephritis was calculated using the mean scores for each pathological feature.

2.2.15 Statistics

Statistical analyses were performed using GraphPad Prism 7.01. Differences between the groups were assessed by One-way ANOVA followed by Tukey's tests to control for type I errors when more than two groups were compared. Student's two-tailed t-tests was used when only two groups were being compared.



Chapter 3

Results

3.1. Differentiation of SLE patient monocytes

3.1.1 Analysis of monocyte populations in SLE patients

Human monocytes are highly heterogeneous and are typically classified into 3 subsets based on surface marker expression and function [85]. The population that expresses CD14 but not CD16 are referred to as classical monocytes. These account for a majority of circulating monocytes. They are highly phagocytic but do not have inflammatory attributes. Monocytes that express both CD14 and CD16 are referred to as intermediate monocytes. These constitute a transitional subset with both phagocytic and inflammatory features. Non-classical monocytes express CD16 and low levels of CD14 and are responsible for the production of inflammatory cytokines upon activation [86].

Samples from 35 SLE patients were analyzed to determine whether their monocyte subpopulations differed from those of normal controls. Samples were derived from patients with both active and inactive disease as determined by the SLEDAI-2K scoring system (Table 1). Patients were treated conventionally, as indicated in Table 1. Monocytes were FACS purified from their PBMC as described in Mat & Met (section 2.). They were stained and classified based on their surface marker expression, as described. Results showed that the total number of monocytes did not differ between SLE patients when compared to healthy controls (Figure 3.1A). However, the relative frequency of non-classical monocytes was significantly decreased while the frequency of inflammatory monocytes was increased (Figure 3.1B). No statistically significant difference in monocyte subtype or frequency was observed when comparing active vs inactive patients (Figure 3.1 C, D).

Table 3. 1: Characteristics of SLE patients.

The SLEDAI-2K scoring system was used to evaluate disease severity. Those with scores >6 were considered to have active disease. Medications included HCQ (hydroxychloroquine), AZA (azathioprine), and MMF (mycophenolate mofetil).

Number of patients	35
Age	46±13
Gender (N and %)	
Female	32 (91%)
Male	3 (9%)
SLEDAI-2K	
<6	30 (85%)
>6	5 (15%)
Medications	
HCQ	27 (77%)
Prednisone	26 (74%)
AZA	7 (20%)
MMF	9 (26%)

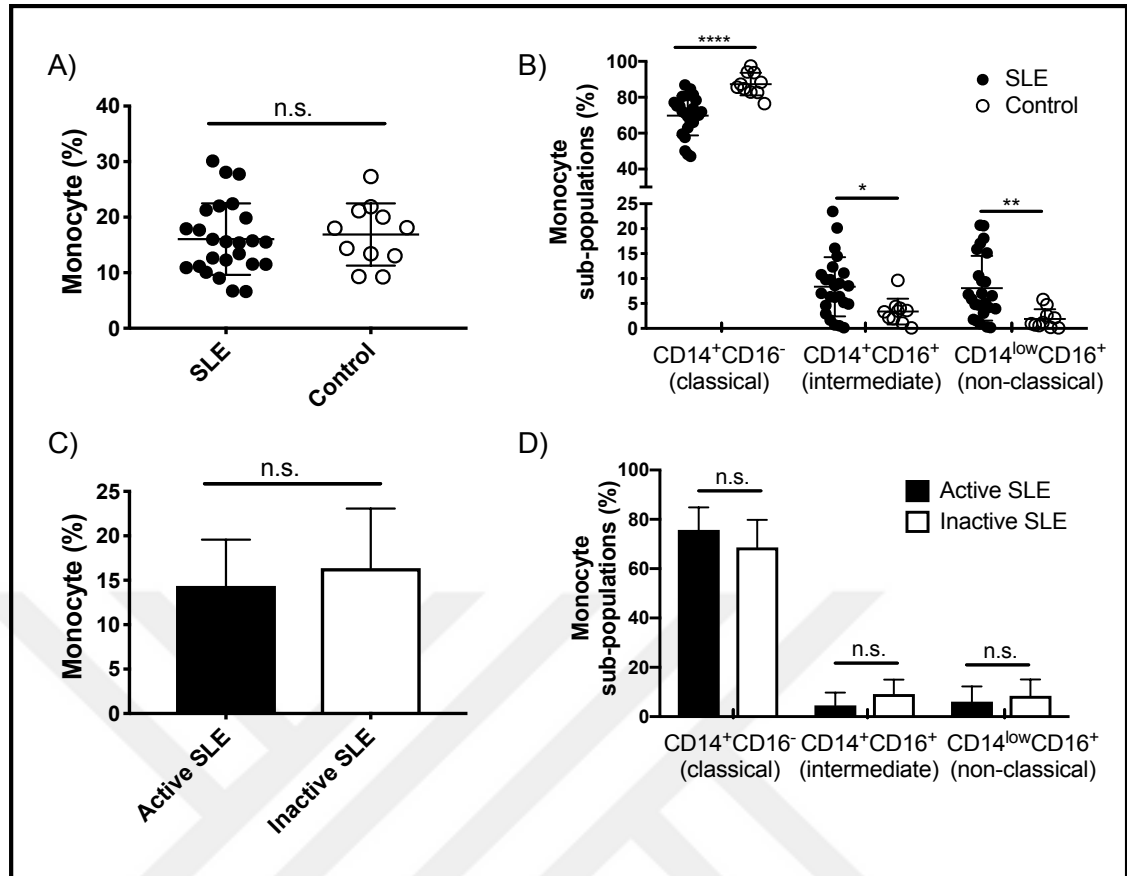


Figure 3.1: Characterization of monocytes from patients with SLE

Data show A) the percent of monocytes in PBMC and B) the percent of each monocyte subpopulation in samples from 24 lupus patients and 10 healthy controls. Comparison of C) total monocyte percentages and D) the percent of each monocyte subpopulation from 5 active and 19 inactive patients. Patients with SLEDAI scores >6 were considered to have active disease. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.1.2 Phenotypic analysis of generated macrophages

Human monocytes can differentiate into macrophage that have different functions, depending on how they are stimulated [80, 96, 263]. These macrophages also differ in their surface phenotype. Macrophage derived from monocytes acquire 25F9, a macrophage activation marker [264]. In the absence of stimulation, the overwhelming majority of monocytes die during culture whereas > 80% monocytes stimulated with $\text{IFN}\gamma$, R848 or MCSF and >60% with PAM3 survive and mature into macrophages (Figure 3.3 A, B). Alternatively activated M2-like macrophages support tissue remodeling and phagocytose apoptotic cells whereas 'classical' pro-inflammatory M1-like macrophages secrete inflammatory cytokines and have anti-tumor effects Those that differentiate into M2-like macrophages can be identified by their expression of CD163, a scavenger receptor [131, 265]. In contrast, M1-like

macrophage express 25F9 but not CD163. >80% of macrophages generated by IFN γ and R848 lacked expression of CD163 and thus could be categorized phenotypically as being M1-like (Figure 3.2 C). When the same monocyte population was stimulated with macrophage colony stimulating factor (MCSF), which is a classical inducer of M2-like macrophages, or the TLR 2/1 agonist PAM3, a majority matured into CD163⁺ 25F9⁺ M2-like macrophages (Figure 3.2 D). There was no difference in the response of monocytes isolated from lupus patients vs normal controls in their response to PAM3, MCSF, R848 or IFN γ . The M2:M1 ratio also showed that MCSF and PAM3 predominantly induced the generation of M2-like macrophages whereas IFN γ and R848 primarily induced M1-like macrophages. (Figure 3.2 E). Although most monocytes from healthy controls die if not stimulated *ex vivo*, those from SLE patients showed a 3-fold improvement in survival and were slightly more likely to differentiate (in the absence of *ex vivo* stimulation) into M1-like macrophages. (p =.031, Figure 3.2 C).

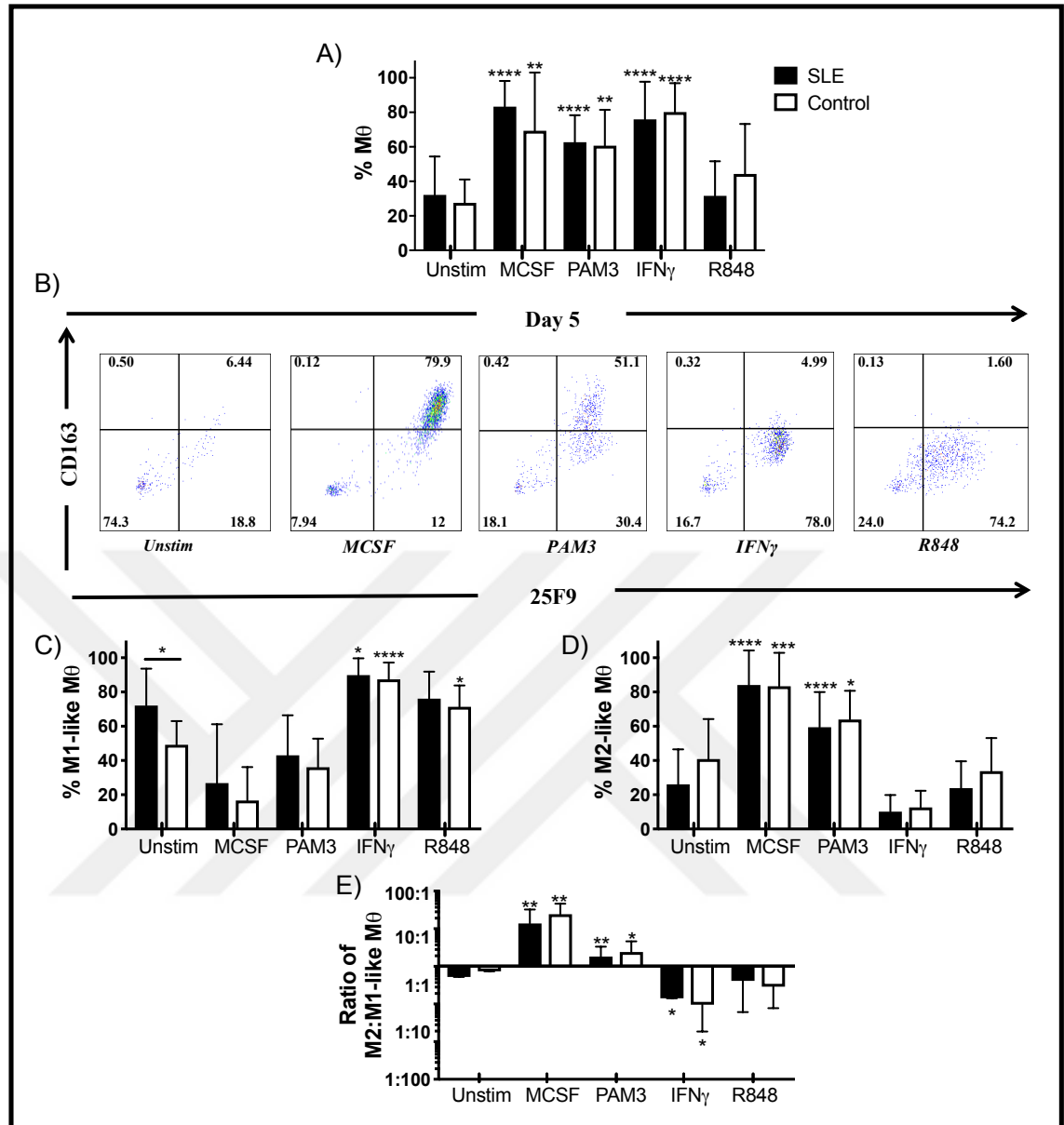


Figure 3.2: Phenotypic analysis of generated macrophages from SLE patient monocytes.

Monocytes were MACS purified from the peripheral blood of lupus patients and normal controls and stimulated in vitro for 5 days with optimal concentrations of MCSF, PAM3, IFN γ or R848. A) The frequency of generated macrophages that acquired 25F9, B) Representative histogram showing the expression of 25F9 and CD163 by cultured cells. Note that <1% of purified monocytes expressed either marker before being placed in culture (day 0), C) percent of 25F9⁺ CD163⁻ M1-like macrophages, D) percent of 25F9⁺ CD163⁺ M2-like macrophages and E) ratio of CD163⁺25F9⁺: CD163⁻25F9⁺ cells. Data derive from independent studies of 23 lupus samples (solid bars) and 9 normal controls (open bars) ** p<.01; *** p<.001; **** p<.0001 compared to unstimulated cells from the same donors.

To further characterize the macrophages generated by PAM3, the expression of surface markers associated with macrophage function was analyzed. These markers

included CD14 and CD44 (whose expression is linked to phagocytosis) and CD40 and CD273 (whose expression is associated with the activation or inhibition of adaptive immune responses, respectively). Results showed that PAM3 and MCSF generated macrophages that expressed high levels of CD14, a myeloid cell marker found primarily on human monocytes. It is also a co-receptor for LPS and has been associated with phagocytosis, a characteristic feature of M2-like macrophages [266]. The MFI values of CD14 expression by macrophages generated after 5 days in culture with MCSF and PAM3 increased 4-6-fold compared to unstimulated controls and 20-30-fold compared to freshly isolated monocytes from SLE patients (Figure 3.3A).

Another surface molecule associated with the phagocytotic clearance of apoptotic cells is CD44. Expression of CD44 was elevated in MCSF and PAM3 generated macrophages from healthy controls (Figure 3.3B). However, in SLE patients, CD44 expression was also upregulated in the phenotypically M1-like macrophages generated by IFN γ and R848. The literature does not help explain this disparity, since there are no previous studies concerning CD44 expression on macrophages generated from lupus monocytes. Experiments performed on normal human macrophages suggest that CD44 can be upregulated by M1 stimuli [267]. According Ruffel et al, despite the association with CD44 expression and apoptosis, there is no correlation between endocytic activity and CD44 expression by M1-like macrophage [268]. Similar to what was found for CD44, M1-like macrophages generated by treating lupus monocytes with IFN γ or R848 expressed significantly elevated levels of CD14 whereas similarly cultured monocytes from healthy controls did not. R848 but not IFN γ generated macrophages from SLE patients significantly increased their CD14 expression when compared to unstimulated controls. This may reflect the ability of R848 to generate a higher frequency of M2-like macrophages (Figure 3.3 A, B).

Along with being up-regulated on M1-like macrophages, CD40 is found on antigen presenting cells and B cells. The interaction of CD40 with its cognate ligand (CD40L) triggers lymphocyte activation, proliferation and initiates immunoglobulin isotype switching. Reports indicate that CD40 expression is increased on macrophages from both SLE and vasculitis patients. As previously reported, M1-

like macrophages generated by IFN γ or R848 selectively up-regulated CD40 expression (Figure 3C) [269]. Higher levels of CD40 were also present on freshly isolated monocytes from SLE patients as previously reported [156, 232].

CD273 (PD-L2) is a ligand for the PD1(programmed cell death-1) protein. CD273 is expressed by DCs, activated monocytes and epithelial cells. It can function as a co-inhibitor of T cell function by acting through PD-1. CD273 is typically expressed by M2-like macrophages generated by MCSF [131, 270]. In normal healthy donors, I found that CD273 expression was increased upon both MCSF and IFN γ stimulation when compared to freshly isolated monocytes (Figure 3.3 D). In lupus monocytes, expression of CD273 was not significantly elevated among MCSF generated macrophages but increased 30-fold in IFN γ generated macrophages (Figure 3.3 D). Previous studies showed that CD273 was not up-regulated by LPS-generated M1-like macrophages but there is no data involving macrophages generated either by IFN γ or R848.

I also analyzed the level of expression of several additional surface markers associated with M2-like macrophage including CD204 (SR-A), CD209 (DC-SIGN), and M1-like macrophage including HLA-DR (antigen presentation) and CD80 (co-stimulatory). However, no significant difference was observed in the expression of these markers in M1- vs M2-like macrophages generated by the stimuli used in this thesis (data not shown).

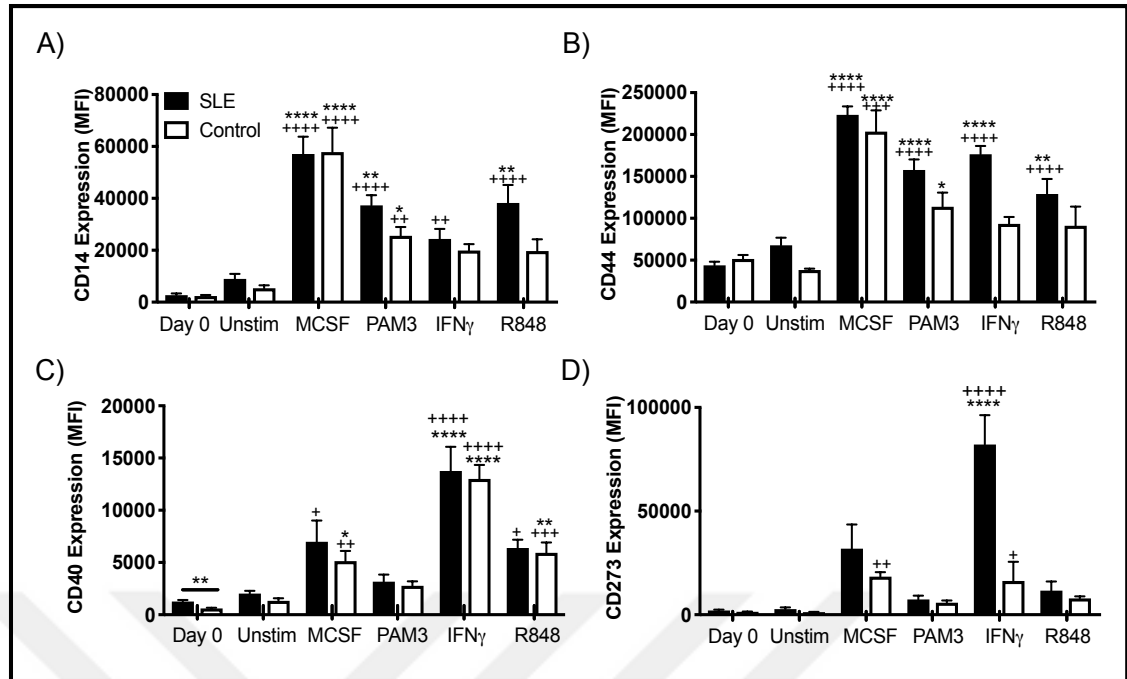


Figure 3.3: Expression of cell surface molecules related with activation and clearance of particles.

Data show MFI of CD14 (A), CD44(B), CD40(C) and CD273(D) expression analyzed by Flow cytometry from 14 SLE patients and 7 healthy controls. ** $p < .01$; *** $p < .001$; **** $p < .0001$ compared to unstimulated cells and ++ $p < .01$; +++ $p < .001$; ++++ $p < .0001$ compared to day 0 from the same donors.

The phenotypes observed in the FACS analysis indicate that PAM3 was as successful as MCSF in terms of generating M2-like macrophages. Since MCSF cannot be used in vivo (as it mediates pleiotropic effects) [82, 271-273], further studies focused on the potential therapeutic utility of PAM3. In these experiments, either IFN γ or R848 was used as controls to generate M1-like macrophages.

3.1.3 Functional assays from generated macrophages

As noted above M1- and M2-like macrophages are distinct in their functional activities. An important feature of M2-like macrophages is their ability to endocytose pathogens and apoptotic cells. Of note, macrophages from SLE patients are impaired in their ability to clear apoptotic cells. The accumulation of degraded self-antigens supports further inflammation and autoantibody production [274, 275]. The endocytic activity of macrophages generated in vitro was evaluated using the zymosan uptake assay. Monocytes were placed in culture with various stimuli for 5 days and then incubated with labeled zymosan particles. The uptake of the particle was examined after 45 minutes by measuring mean fluorescence intensity

(normalized by subtracting background values from unstimulated macrophages). Results show that both lupus and control macrophages generated by PAM3 acquired endocytic activity (reflected by their ability to internalize zymosan particles) whereas those treated with M1 stimuli did not (Figure 3.4 B). In both lupus patients and controls, this gain in function correlated with increased expression of CD163 by PAM3 treated monocytes ($R^2 = 0.92$, Figure 3.4 C).

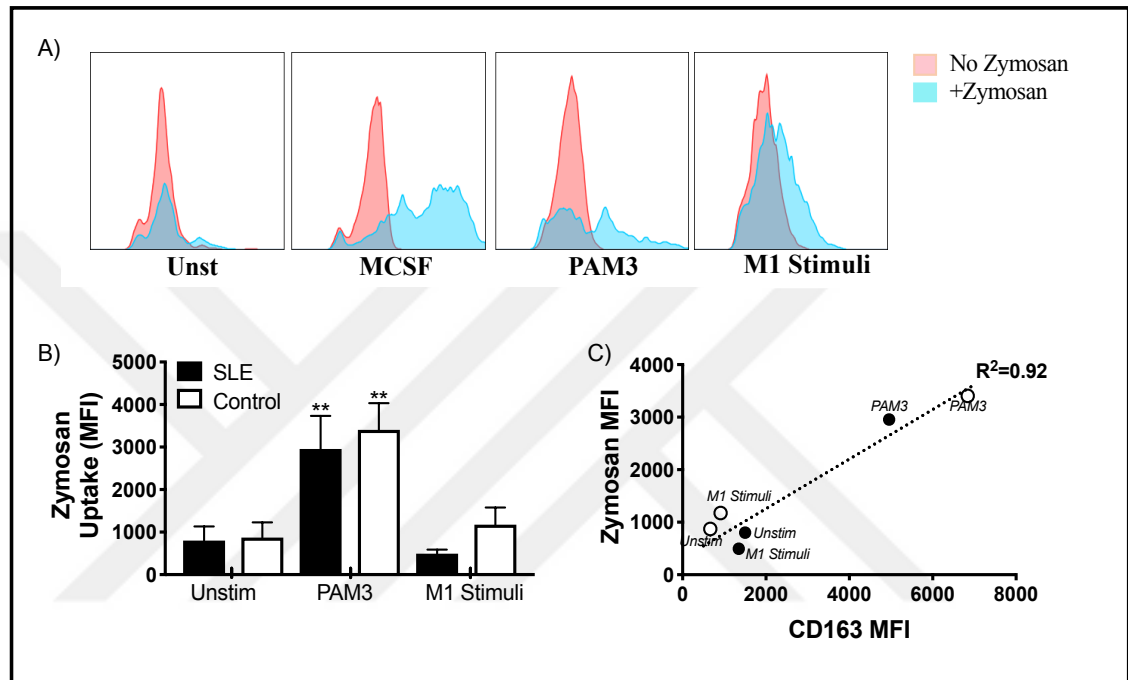


Figure 3.4: Endocytic ability of generated macrophages.

MACS purified monocytes were cultured as described in Fig 2. A) Representative histogram showing the uptake of zymosan particles B) Zymosan Uptake MFI of labelled particles after 5 days of culture (N=17 lupus samples and 6 controls). C) Correlation between the expression of CD163 and uptake of zymosan particles. ** $p < .01$; *** $p < .001$; **** $p < .0001$ compared unstimulated cells from the same donors. IFN γ or R848 was used as M1 stimuli

M1-like macrophages produce pro-inflammatory cytokines such as TNF α and IL-12 that help protect the host from infection. Yet the increased production of pro-inflammatory cytokines is associated with disease flares in lupus patients [276]. The cytokine profile of macrophages generated in vitro was monitored by ELISA and by intracytoplasmic cytokine staining. ELISA results showed that TNF α levels were increased in macrophages generated by M1-stimuli. Similarly, the frequency of IL12 $^{+}$ cells was increased (Figure 3.5 A, B). In contrast, neither the amount nor frequency of macrophages producing these cytokines was significantly elevated in

cultures supplemented with PAM3. Rather, increased levels of anti-inflammatory IL-10 was detected in cultures arising from PAM3 generated macrophages (Figure 3.5 C).

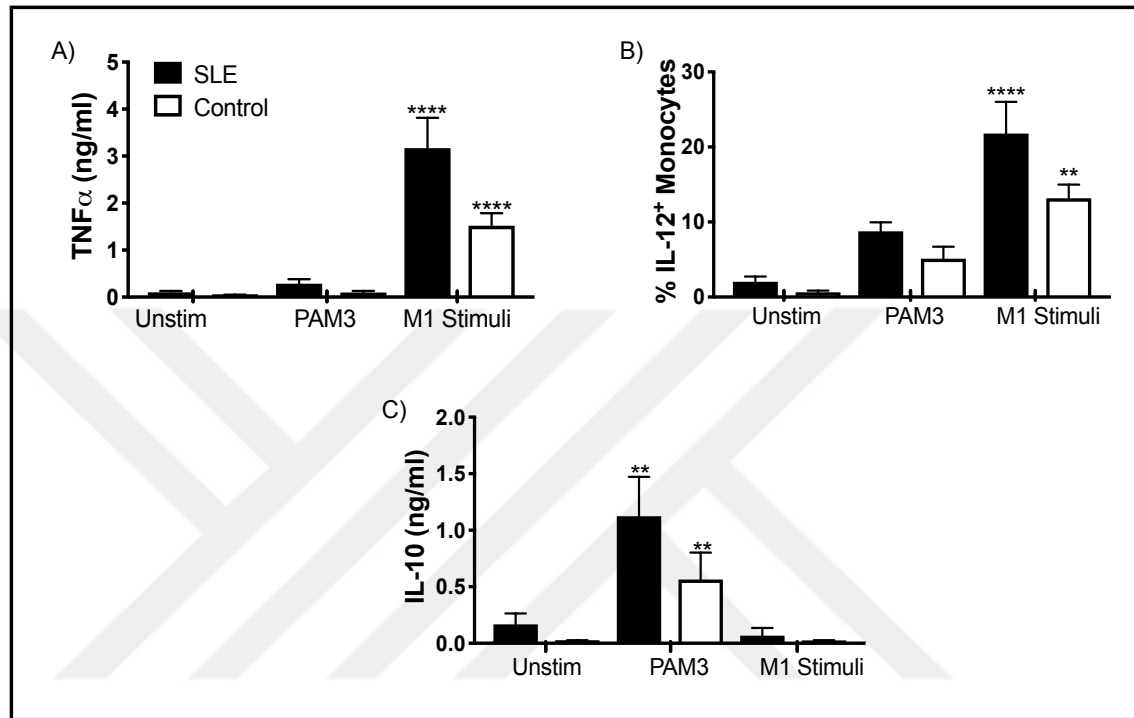


Figure 3.5: Cytokine secretion profile of generated macrophages. .

A) Concentration of TNFα in culture supernatants. B) Percent of cultured cells staining for intracytoplasmic IL-12. C) Concentration of IL-10 in culture supernatants (N=12 lupus samples and controls). ** p<.01; *** p<.001; **** p<.0001 compared unstimulated cells from the same donors. IFNγ or R848 was used as M1 stimuli.

3.2. Differentiation of Vasculitis Patient monocytes

3.2.1 Analysis of Monocyte populations in Vasculitis patients

Experiments similar to those performed on samples from SLE patients were conducted on PBMC obtained from 9 patients with vasculitis, 8 of whom had Giant Cell Arthritis (Table 2). PBMC from vasculitis patients had significantly elevated percentages of monocytes, a finding consistent with previous reports (Figure 3.6 A, [252]). The frequency of inflammatory monocytes was significantly elevated whereas those with a non-inflammatory (classical) monocyte phenotype were decreased when compared to healthy controls (Figure 3. 6 B)

Table 3.2: Characteristics of Vasculitis patients
Giant Cell Arthritis (GCA) and Takayasu's Arthritis (TAK)

Number of patients	10
Age	71±10
Gender (N and %)	
Female	7 (70%)
Male	3 (30%)
Type	
GCA	9(90%)
TAK	1(10%)
Disease Severity	
Active disease	3(30%)
Remission	7(70%)
Medications	
None	4(40%)
Immunosuppressive drugs	6(60%)

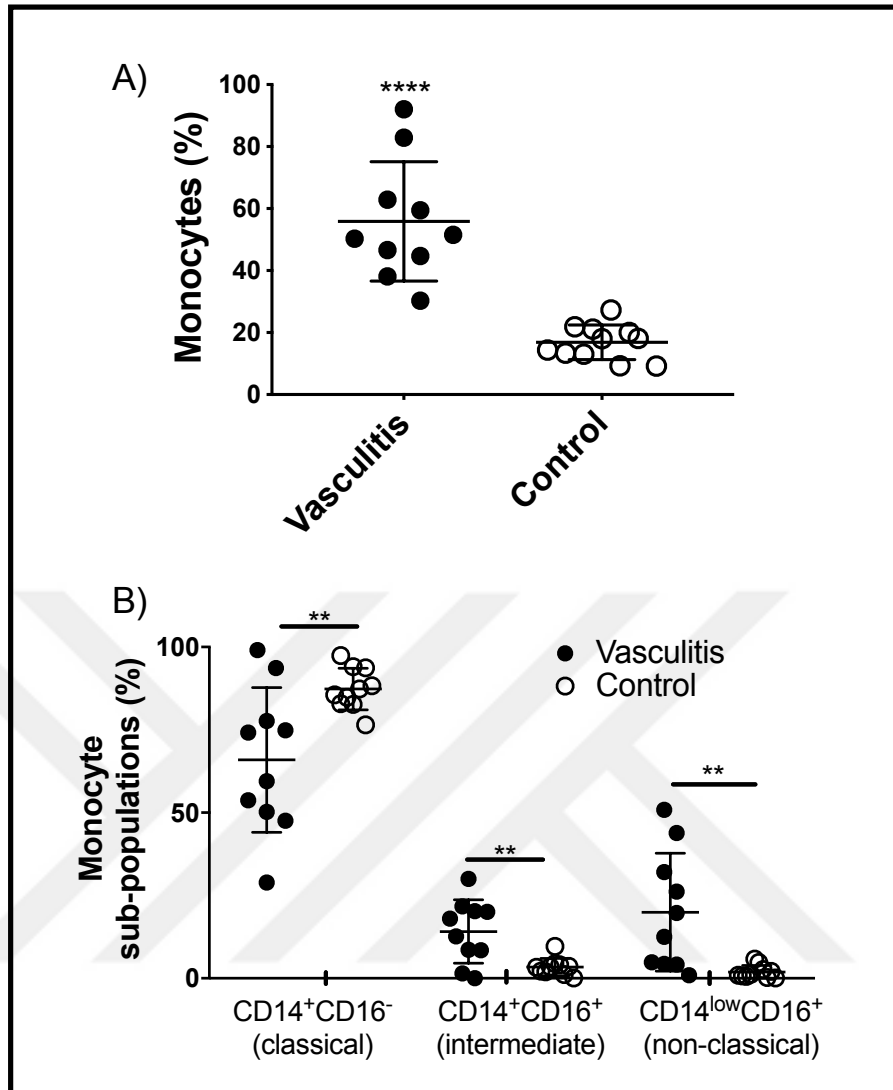


Figure 3.6: Characterization of monocytes from patients with Vasculitis.

Data show A) the percent of monocytes in PBMC and B) the percent of each monocyte subpopulation in samples from 9 vasculitis patients and 10 healthy controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2.2 Phenotypic analysis of generated macrophages

Sorted monocytes were cultured in vitro for 5 days in the presence of agents that induce differentiation into M1- or M2-like macrophages. $\text{IFN}\gamma$ and R848 were used to generate M1-like macrophages whereas MCSF and PAM3 were used to produce

M2-like macrophages. FACS analysis of the cultured cells showed that a majority (60 - 80%) acquired expression of the macrophage activation marker 25F9 (Figure 3.7 A). In the absence of in vitro stimulation, vasculitis monocytes preferentially differentiated into M1-like macrophages when compared to monocytes from healthy

controls ($p=0.01$, Figure 3.7 B). MCSF and PAM3 induced $>50\%$ of vasculitis monocytes to differentiate into M2-like macrophages characterized by the expression of CD163 (Figure 3.7C). The frequency of M2-like macrophages generated by both stimulants were not significantly different from healthy controls. As expected, $\text{IFN}\gamma$ induced the generation of M1-like macrophages determined by expression of 25F9 but not CD163. However, R848 stimulation resulted in a mixed population of M1 and M2-like macrophages, thus it was not a strong inducer of M1 like macrophages like $\text{IFN}\gamma$ (Figure 3.7 B). M2:M1 ratios, calculated as the number of 25F9⁺ cells expressing CD163 divided by CD163⁻ cells, showed that MCSF and PAM3 induced primarily M2-like macrophages while $\text{IFN}\gamma$ and R848 generated M1-like macrophages, consistent with previous findings from lupus and control samples (Figure 3.7 D).

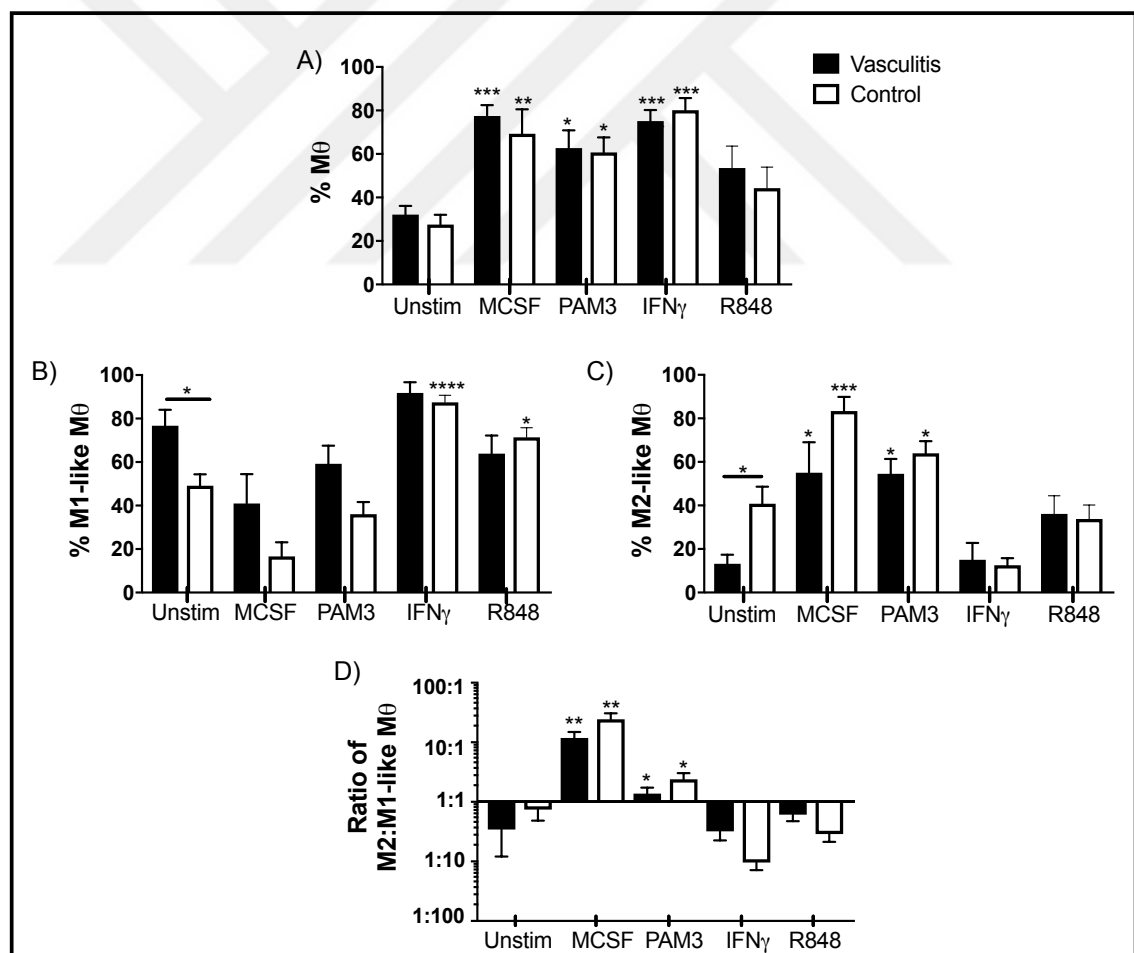


Figure 3.7: Phenotypic analysis of generated macrophages from Vasculitis Patient monocytes.

Monocytes were MACS purified from the peripheral blood of vasculitis patients and normal controls and stimulated *in vitro* for 5 days with optimal concentrations of PAM3, M-CSF, IFN γ or R848. A) Percent of cultured cells that acquired 25F9 expression, B) percent of 25F9⁺ CD163⁻ M1-like macrophages, C) percent of 25F9⁺ cells that co-expressed the CD163 M2-like macrophage marker and D) ratio of CD163⁺25F9⁺: CD163⁻25F9⁺ cells. Data derive from independent studies of 9 vasculitis samples (solid bars) and 9 normal controls (open bars) where R848 or IFN γ were used as the M1 stimuli. ** p<.01; *** p<.001; **** p<.0001 compared to unstimulated cells from the same donors

The same surface markers used to examine the maturation of lupus monocytes in section 3.3 were used to study macrophage behavior in monocytes from patients with vasculitis. In contrast to the results obtained from lupus monocytes, increased expression of CD14 was observed after MCSF but not PAM3 treatment of vasculitis monocytes (Figure 3.8 A). This difference could not be attributed to variation in the ability of PAM3 to generate M2-like macrophages from lupus vs vasculitis monocytes (Figure 3.2 D and Figure 3.7 C). On the other hand, unstimulated monocytes from vasculitis patients had 1.5-fold higher levels of CD14.

Analysis of CD44 expression indicated that monocytes from vasculitis and lupus patients responded similarly to *ex vivo* stimulation. Expression of CD44 was up-regulated in both M2 and M1-like macrophages when compared to freshly isolated monocytes (Figure 3B). This effect reached statistical significance for MCSF and IFN γ treated cultures when compared to unstimulated cells cultured for 5 days (Figure 3.8 B). In healthy controls, CD44 was elevated primarily among M2-like macrophages generated by MCSF and PAM3.

CD40 was found to be significantly up-regulated in unstimulated cultures of vasculitis monocytes when compared to healthy controls. This correlated with the increased frequency of M1-like macrophages in unstimulated cultures of those cells (Figure 3.7.C). CD40 expression was significantly elevated among IFN γ -induced macrophages from both vasculitis patients and healthy controls. CD40 was generally present on M1-like macrophages although the difference between M1- vs M2-like macrophages did not reach statistical significance in R848 generated macrophages from vasculitis patients, which correlated with the finding that R848 was not as efficient as IFN γ in the generation of M1-like macrophages (Figure 3.7 B).

CD273 expression, even though it is generally used to discriminate M2-like macrophages, was enhanced on vasculitis patient macrophages generated by IFN γ similar to lupus monocyte cultures, whereas in healthy controls CD273 was increased by both MCSF and IFN γ when compared to day 0 monocytes (Figure 3.8 C).

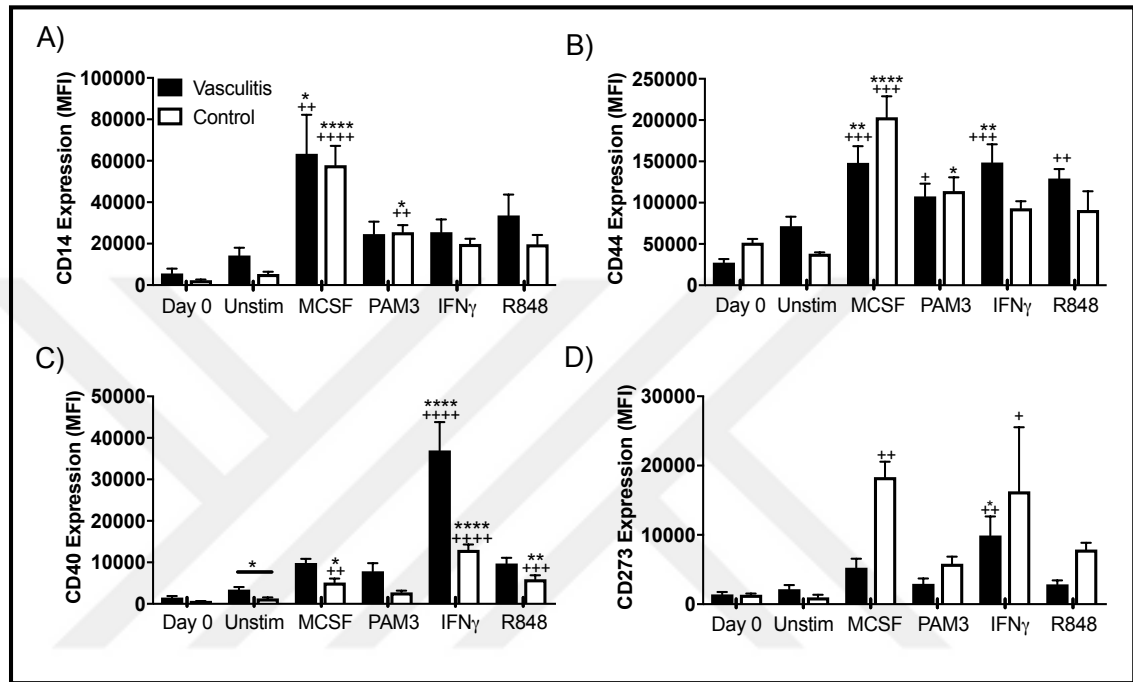


Figure 3.8: Expression of cell surface molecules related with activation and clearance of particles.

Data show MFI of CD14 (A), CD44(B), CD40(C) and CD273(D) expression analyzed by Flow cytometry from 8 Vasculitis patients and 7 healthy controls. ** p<.01; *** p<.001; **** p<.0001 compared to unstimulated cells and ++ p<.01; +++ p<.001; ++++ p<.0001 compared to day 0 from the same donors.

PAM3 was as successful in generating M2-like macrophages from monocytes derived from vasculitis patients as it was for SLE samples. Further studies thus focused on comparing the activity of PAM3 to IFN γ or R848 (which preferentially generate M1 macrophages).

3.2.3 Functional assays from generated macrophages

The endocytic ability of PAM3 generated macrophages was measure by Zymosan uptake (as previously described, section 3.1.3). Consistent with earlier findings, macrophages generated by PAM3 had higher endocytic ability than those derived from stimuli that preferentially generate M1-like macrophages (Figure 3.9 A). This

increased ability was correlated with the expression of CD163 ($R^2=0.89$, Figure 3.9B).

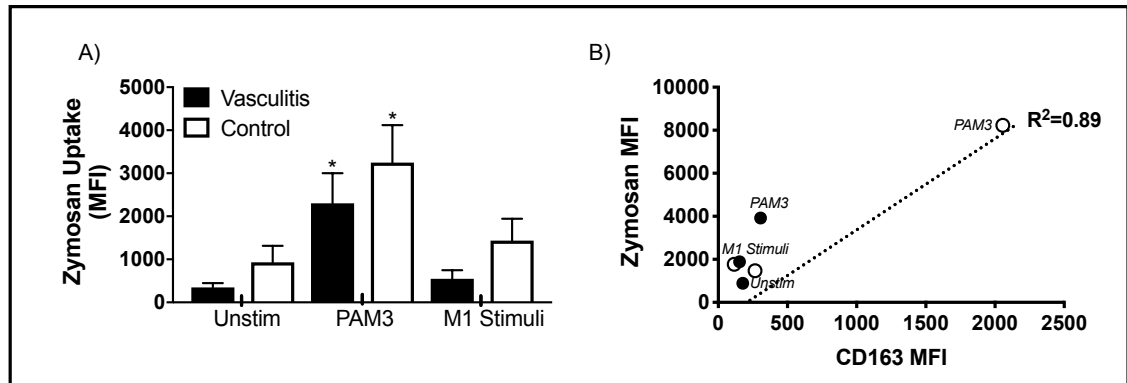


Figure 3.9: Endocytic abilities of generated macrophages. .

MACS purified monocytes were cultured as described in Fig 2. A) The uptake of zymosan-labelled particles after 5 days of culture (N= 10 vasculitis samples and 7 controls). B) Correlation between the expression of CD163 and uptake of zymosan particles. ** $p<.01$; *** $p<.001$; **** $p<.0001$ compared unstimulated cells from the same donors.

Cytokine secretion was investigated as a function of M1-and M2-like macrophages. M1-like macrophages are known produce pro-inflammatory cytokines like $\text{TNF}\alpha$ and IL-12 whereas M2-like macrophages secrete anti-inflammatory cytokines such as IL-10. $\text{TNF}\alpha$, IL-12 and IL-10 levels were measured in the supernatant of macrophage cultures by ELISA. M1-stimuli generated macrophages secreted high levels of both $\text{TNF}\alpha$ and IL-12 (Figure 3.10 A, B). PAM3 generated macrophages did not increase their secretion of inflammatory cytokines but did significantly elevate production of anti-inflammatory IL-10 (Figure 3.10 C).

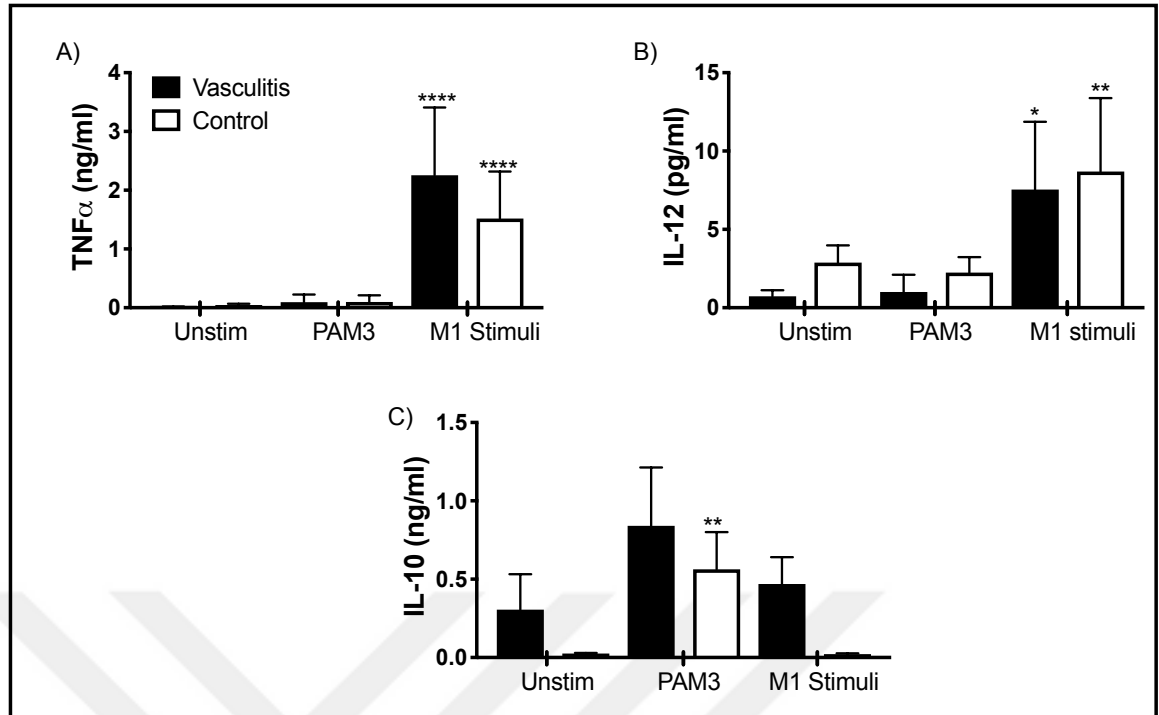


Figure 3.10: Cytokine secretion profile of generated macrophages.

Concentration of TNF α (A), IL-12(B) and IL-10 (C) in culture supernatants. B (N = 8 vasculitis samples and controls). ** p<.01; *** p<.001; **** p<.0001 compared unstimulated cells from the same donors.

3.3. Differentiation of Myositis Patient monocytes

3.3.1 Analysis of Monocyte populations in Myositis patients

The effect of stimulating monocytes from patients with myositis was examined. Patient information is provided in Table 3. Monocytes were sorted from PBMCs as they were from lupus and vasculitis samples. Monocyte sub-populations were characterized based on surface marker expressions. There was no significant difference in the total monocyte percentages between patients and controls (Figure 3.11 A). However, similar to the results obtained from lupus and vasculitis patients, intermediate monocytes with inflammatory attributes were increased, and ‘non-inflammatory’ classical monocytes were decreased in myositis patients when compared to healthy controls (Figure 3.12 B).

Table 3.3: Characteristics of Myositis patients.

Number of patients	12
Gender (N and %)	
Female	10 (83%)
Male	2 (17%)
Adult	5 (42%)
Juvenile	7(58%)
Dermatomyositis	11 (92%)
Polymyositis	1 (8%)
Score	
MMT8	
Adult	72.7±7.8
Juvenile	71.5±11.6
Medications	
Corticosteroids	12 (100%)
NSAID	7 (58%)
HCQ	7 (58%)
MMF	6 (50%)

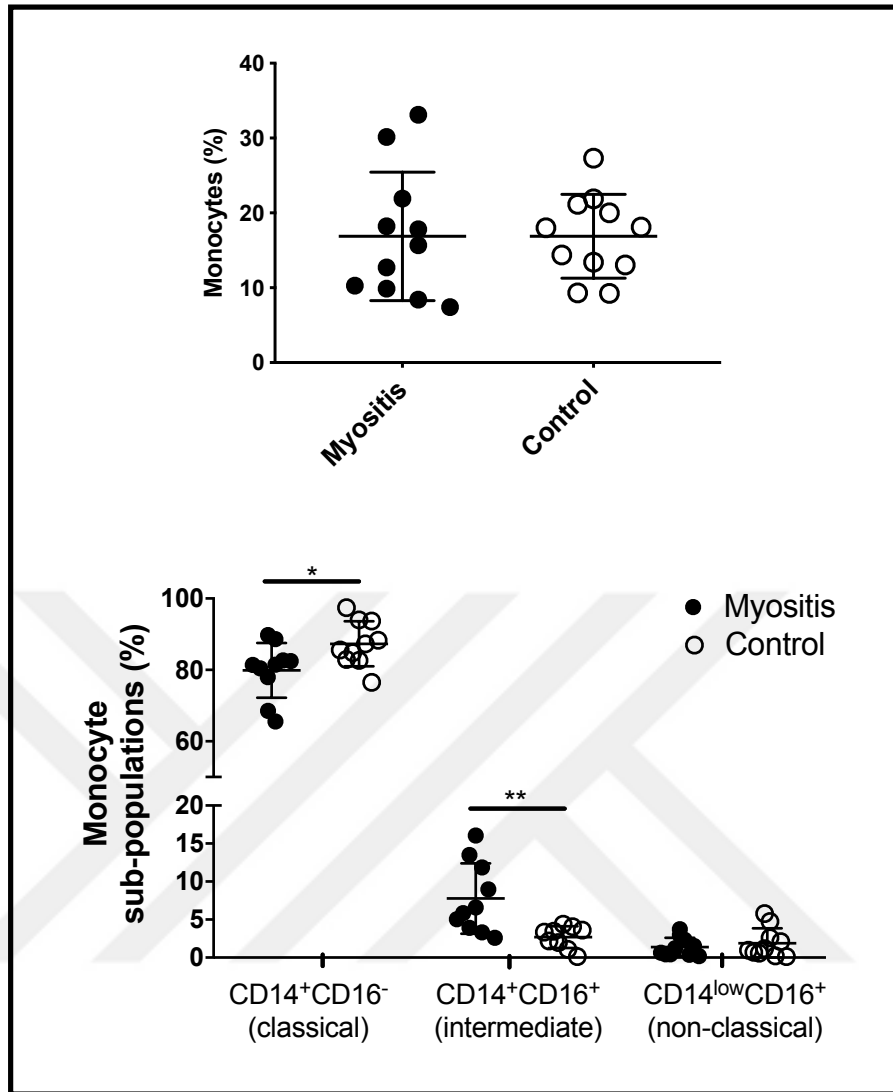


Figure 3.11: Characterization of monocytes from patients with Myositis.

Data show A) the percent of monocytes in PBMC and B) the percent of each monocyte subpopulation in samples from 11 myositis patients and 11 healthy controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3.2 Phenotypic analysis of generated macrophages

The same culture protocol was used to mature monocytes from myositis patients as described above for lupus and vasculitis samples. <60% of the monocytes from both myositis and controls incubated with MCSF, PAM3 and $\text{IFN}\gamma$ and 50% of the monocytes incubated with R848 differentiated into macrophages as reflected by 25F9 expression (Figure 3.12 A). In the absence of stimulation only >20% of monocytes acquired 25F9, a finding consistent with results from SLE and vasculitis patients (Figure 3.12 A). >80% of the macrophages generated by MCSF expressed CD163 and thus were phenotypically M2-like macrophages whereas with PAM3

≈60% expressed CD163 (Figure 3.12C). There was no significant difference in the ability of MCSF vs PAM3 to generate M2-like macrophages comparing myositis patients to healthy controls. As expected IFN γ and R848 induced macrophages that lack the expression of CD163, defining them as M1-like macrophages.

Similar to unstimulated SLE and vasculitis monocytes, although most unstimulated myositis monocytes died during culture, those that did differentiate selectively generated M1-like macrophages ($p < 0.0001$ compared to control, Figure 3.12B)

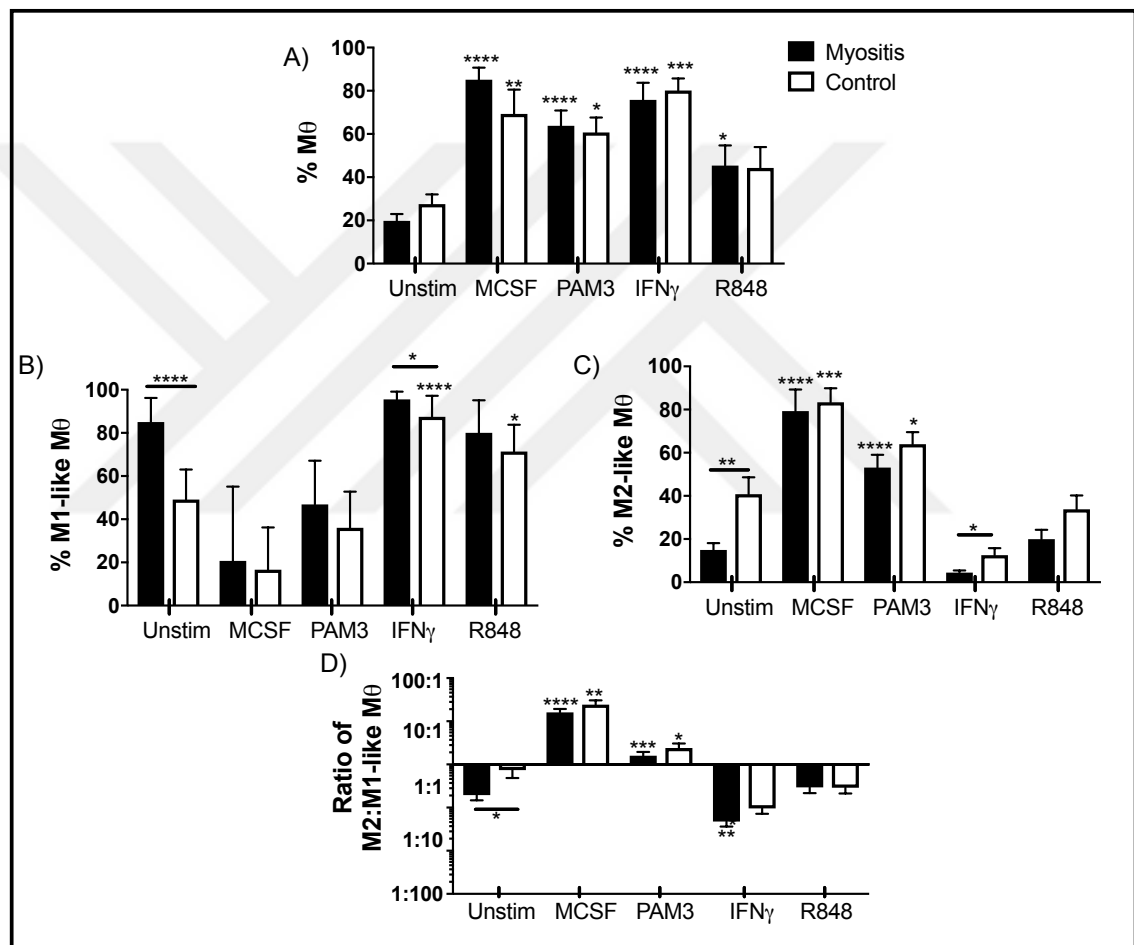


Figure 3.12: Phenotypic analysis of generated macrophages from Myositis Patient monocytes.

Monocytes were MACS purified from the peripheral blood of vasculitis patients and normal controls and stimulated in vitro for 5 days with optimal concentrations of PAM3, M-CSF, IFN γ or R848. A) Percent of cultured cells that acquired 25F9 expression, B) percent of 25F9+ CD163- M1-like macrophages, C) percent of 25F9+ cells that co-expressed the CD163 M2-like macrophage marker and D) ratio of CD163+25F9+: CD163-25F9+ cells. Data derive from independent studies of 12 myositis samples (solid bars) and 9 normal controls (open bars) ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to unstimulated cells from the same donors.

Although disease manifestations are similar between patients with adult vs juvenile myositis, there are some differences between them. The literature indicates that juvenile and adult myositis differ in disease progression. Adults are at increased risk of malignancy and interstitial lung disease which is not the case for juveniles who have a better long-term prognosis and survival rate. Seven of the myositis samples were from those with juvenile disease while four were from patients with adult myositis (AM). While there was no difference in the total frequency of monocyte between these two groups (Figure 3.13A) a significant increase in the ‘inflammatory’ intermediate monocyte subpopulation was observed in AM vs JM samples (Figure 3.13 B). Moreover, AM patients had significantly higher frequencies of M1-like macrophages than those with JM (Figure 3.13 C). Unfortunately, this difference did not correlate with age, muscle weakness score, medication or the presence of autoantibody. It is interesting to consider whether the differences in inflammatory monocytes/macrophages might be a reflection or contributor to differences in disease progression.

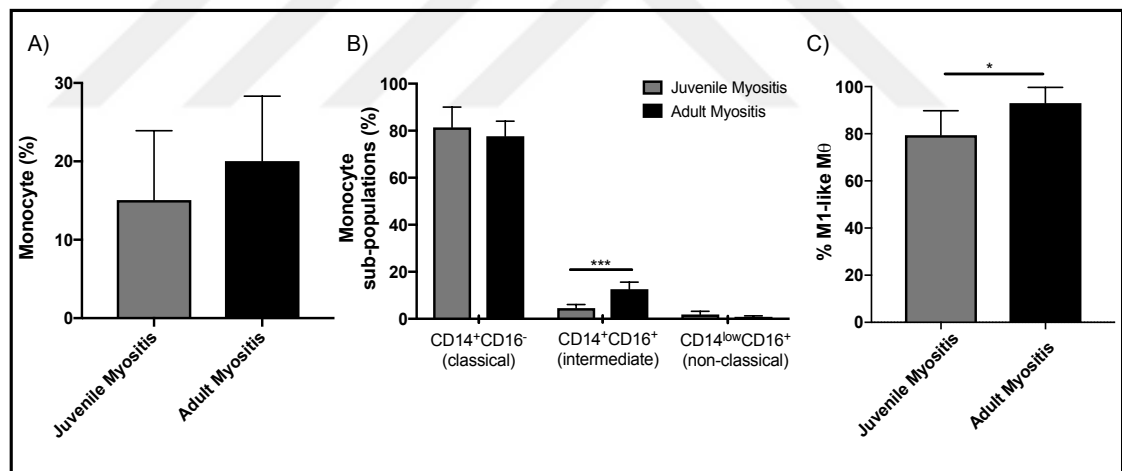


Figure 3.13.: Adult myositis patients have higher inflammatory monocytes and macrophages when compared to juvenile.

A) Total monocyte frequencies and B) Monocyte subpopulations compared from AM and JM patients as a fraction of PBMC. C) Frequency of M1-like macrophages characterized by the expression of 25F9, generated in the absence of any stimulants. Adult myositis (AM) N= 4, Juvenile Myositis (JM) N=7, ** p<.01; *** p<.001; **** p<.0001 compared to unstimulated cells

Cell surface markers were used to analyze the phenotype of PAM3 generated macrophages from myositis patients. There was a significant increase in CD14 expression upon MCSF stimulation from myositis monocytes. CD14 expression did

not increase significantly after PAM3 stimulation in contrast to the effect observed on lupus monocytes (Figure 3.14 A). On the other hand, the expression of CD44 increased significantly, similar to results from lupus and vasculitis patients (Figure 3.14 B). Expression of CD40 and CD273 reached significant levels in macrophages generated only by IFN γ , again consistent with results from lupus and vasculitis patients (Figure 3.14 C, D).

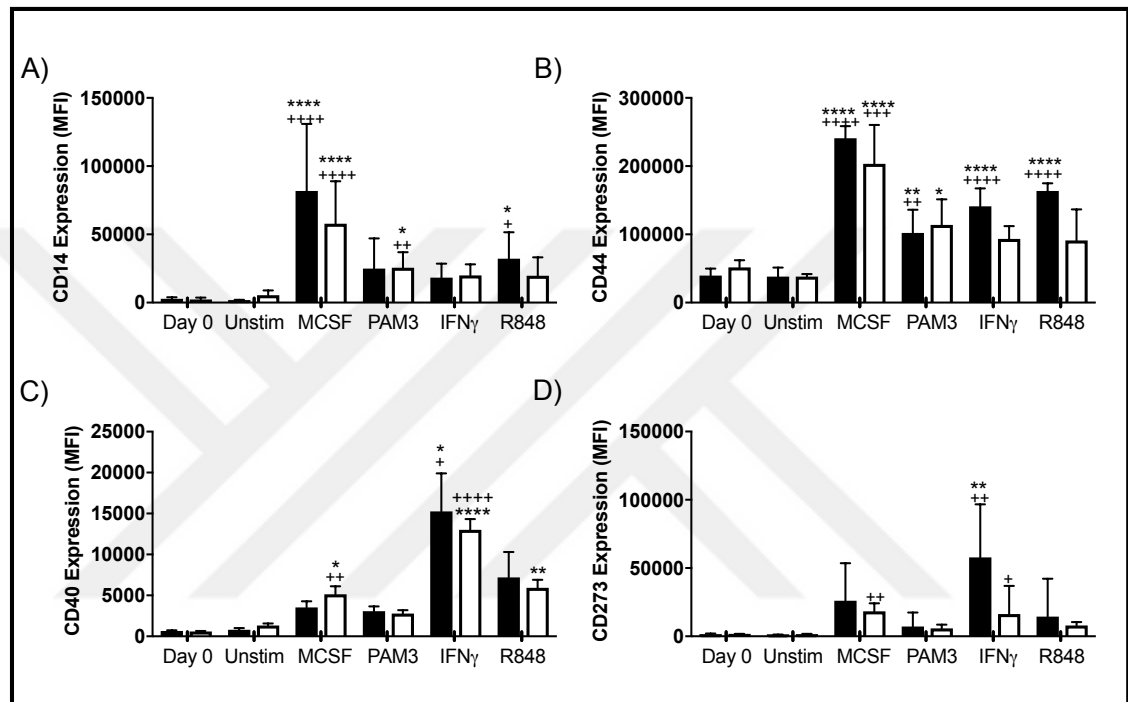


Figure 3.14: Expression of cell surface molecules related with activation and clearance of particles.

Data show MFI of CD14 (A), CD44(B), CD40(C) and CD44(D) expression analyzed by Flow cytometry from 4 Myositis patients and 7 healthy controls. ** p<.01; *** p<.001; **** p<.0001 compared to unstimulated cells and ++ p<.01; +++ p<.001; ++++ p<.0001 compared to day 0 from the same donors.

3.3.3 Functional assays from generated macrophages

Macrophages generated from myositis monocytes by PAM3 acquired high endocytic activity (a characteristic feature of M2-like macrophages) as determined by Zymosan uptake assay. The endocytic ability of PAM3 generated macrophages correlated with the expression of CD163 ($R^2=0.72$, Figure 3.15 B). M1-like macrophages lack this ability (Figure 3.15A).

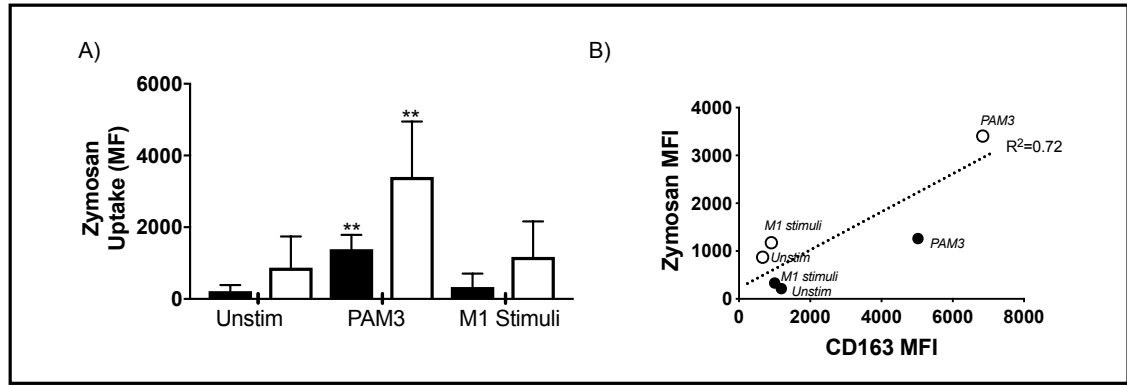


Figure 3.15: Endocytic abilities of generated macrophages.

MACS purified monocytes were cultured as described in Fig 2. A) The uptake of zymosan-labelled particles after 5 days of culture (N= 3 myositis samples and 7 controls). B) Correlation between the expression of CD163 and uptake of zymosan particles. ** $p<.01$; *** $p<.001$; **** $p<.0001$ compared unstimulated cells from the same donors.

One of the defining functions of M1-like macrophages is their ability to secrete inflammatory cytokines. Myositis monocytes stimulated to generate M1-like macrophage produced increased amounts of $\text{TNF}\alpha$ as determined by cytokine ELISA of culture supernatants (Figure 3.16A). Intracytoplasmic staining confirmed that M1-like macrophages produced inflammatory cytokines such as IL12 (Figure 3.16 B). By comparison, PAM3 generated macrophage produced the anti-inflammatory IL-10 (Figure 3.16 C).

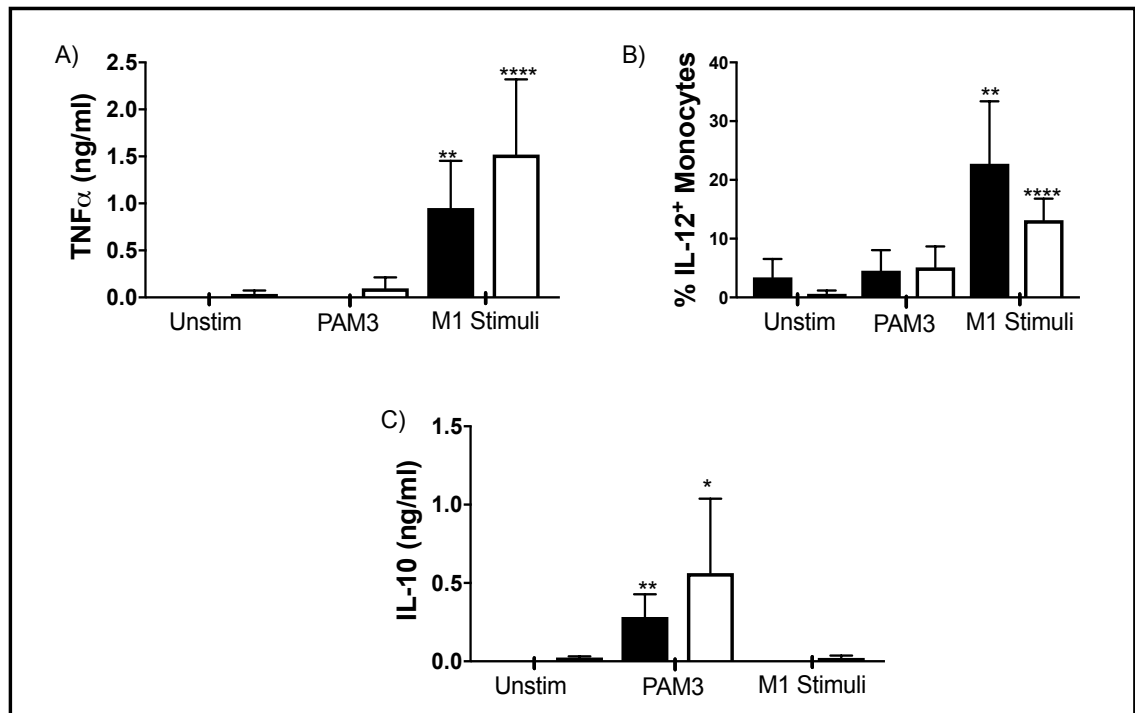


Figure 3.16: Cytokine secretion profile of generated macrophages.

A) Concentration of TNF α in culture supernatants. B) Percent of cultured cells staining for intracytoplasmic IL-12. C) Concentration of IL-10 in culture supernatants (N=4 myositis samples and controls). ** p<.01; *** p<.001; **** p<.0001 compared unstimulated cells from the same donors. IFN γ or R848 was used as M1 stimuli.

Phenotypic and functional analyses showed that PAM3 was successful in generating M2-like macrophages from both SLE, vasculitis and myositis patient monocytes.

As there is no murine model of vasculitis and not yet a widely accepted myositis model, my subsequent studies focused on investigating the therapeutic activity of PAM3 in vivo using a well-established murine model of SLE.

3.4 Therapeutic effects of PAM3 treatment in a mice model of lupus

Female NZB x NZW F1 (hereafter NZB/W) mice develop lupus that resembles human disease both immunologically and clinically. The NZB/W mice were treated with 100 μ g of PAM3 weekly, a dose previously reported to be effective in the treatment of allergic asthma and cardiac dysfunction [260-262]. PAM3 treatment was initiated either before the initiation of kidney damage at 7 weeks of age ("early"), or after the onset of detectable kidney damage as determined by increased proteinuria at 13 weeks of age ("late", p .02, data not shown).

3.4.1 Analysis of the macrophage phenotype from NZB x NZW F1

To evaluate the effect of repeated PAM3 treatment on the phenotype of macrophage present in NZB/W mice, peritoneal cells were collected, stained, and analyzed by FACS. Macrophages were identified based on their co-expression of CD11b and F480. The mannose receptor CD206 was used to distinguish between M1 and M2 macrophages (Figure 3.17 A, B). The frequency of CD206⁺ M2 macrophage was significantly reduced in control NZB/W mice (injected only with PBS) when compared to BALB/c controls (p< 0.0001, Figure 3.17A) indicating that the untreated NZB/W mice have higher frequencies of M1 macrophages supporting previous evidence [277]. Treatment with PAM3 started either early or late in the disease process resulted in a significant increase in the frequency of CD206⁺ M2 macrophages (Figure 3.17A). The ratio of M1:M2 macrophages was determined by

dividing CD206⁺F480⁺ M1 cells by CD206⁺ F480⁺ M2 cell numbers. Early and late PAM3 treatment both resulted in the generation of more M2 macrophages, leading to a normalization of the M1:M2 ratio (Figure 3.17 B). While there was no significant difference in the M1:M2 ratio of mice treated early vs late with PAM3, the effect of early treatment was more significant when compared to the PBS controls (Figure 3.17 B).

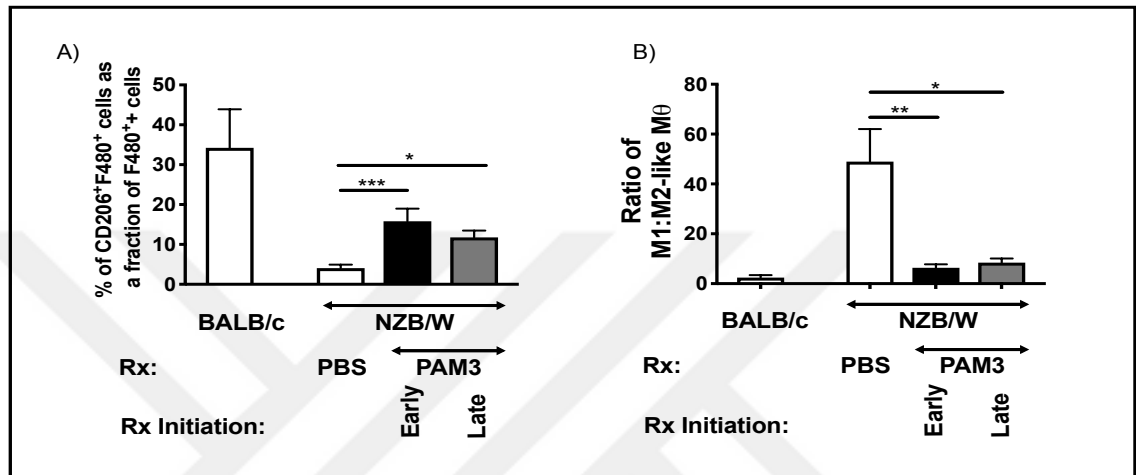


Figure 3.17: Effect of PAM3 treatment on the M1:M2 ratio of NZB/W mice.

Female NZB/W mice were treated weekly with 100 μ g of PAM3 starting either early (7 wk) or late (13 wk). Controls included littermates treated with PBS and normal (BALB/c) mice. Data show, A) the frequency of CD206⁺ F480⁺ macrophages as a fraction of total macrophages, B) M1:M2 ratio, calculated based on the ratio of CD206⁻:CD206⁺ F480⁺ macrophages collected from the peritoneal cavity at 6 mos of age. N = 5 NZB/W mice/group, N = 2 BALB/c controls. * p < 0.05; ** p < 0.01; *** p < 0.001 for PAM3 vs PBS treated NZB/W mice.

3.4.2. Effect of PAM3 on the immune manifestation of lupus

NZB/W mice develop multiple immune manifestation of lupus including the production of autoAbs and inflammatory cytokines [164]. The serum level of IgG anti-dsDNA was measured using ELISA. Results indicate that both early & late PAM3 treatment reduce the level of these autoAb by 3-4-fold (Figure 3.18 A). This decrease was accompanied by a significant reduction in IL-12 levels in the serum of PAM3 treated mice (Figure 3.18 B). TNF α and IL-10 levels were not detectable in mouse serum (data not shown). Thus, supernatants from splenocyte cultures were used to measure the secretion of TNF α and IL-10. The amount of TNF α spontaneously being secreted ex vivo by splenocytes was also reduced in the PAM3 treated mice (Figure 3.18 C). Conversely, IL-10 levels were significantly increased.

The level of IL-10 was elevated in both early and late treated mice but this effect did not reach statistical significance in the late treatment group (Figure 3.18 D).

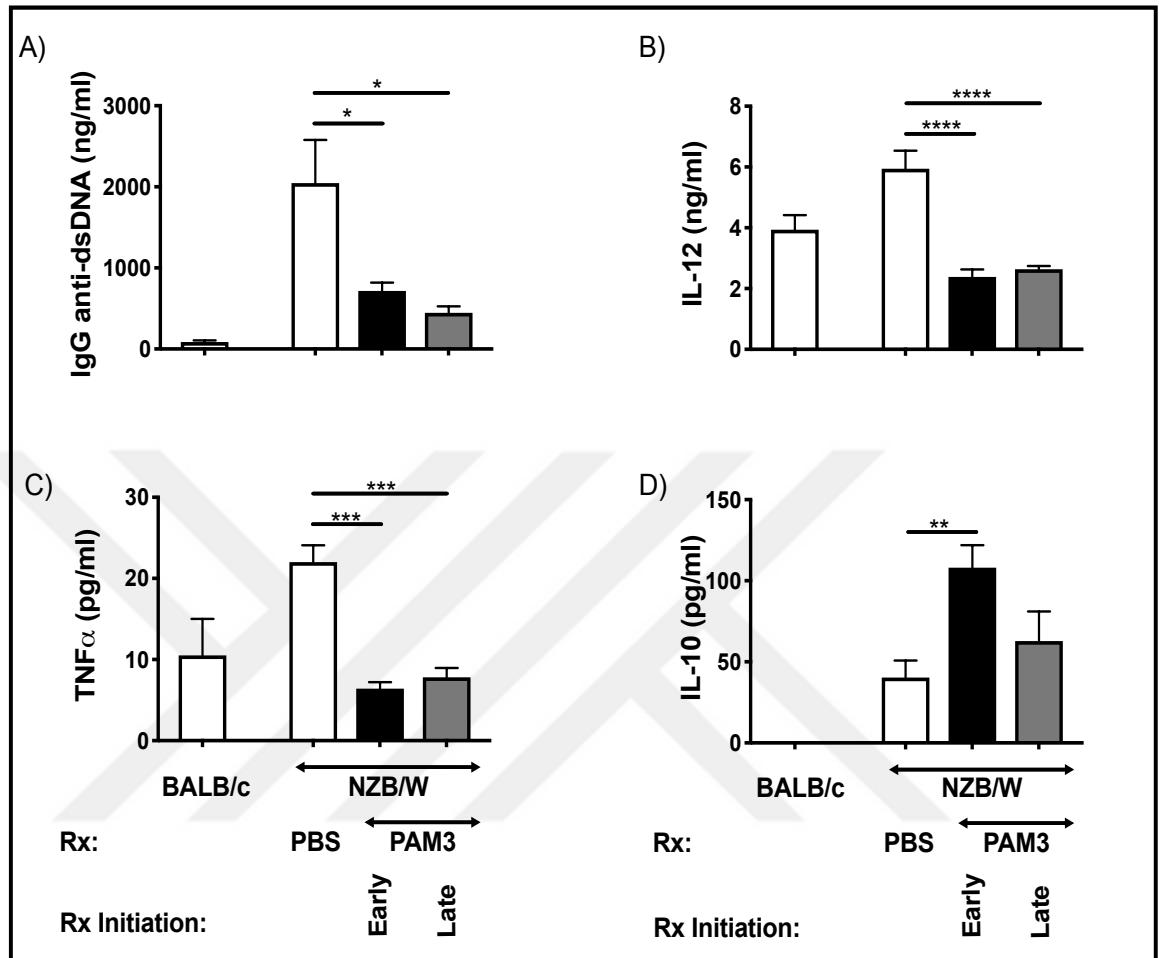


Figure 3.18: Effect of PAM3 treatment on the immune manifestations of lupus. Mice were treated as described in Fig 4. Serum collected at 6 mos was analyzed for (A) IgG anti-dsDNA and (B) IL-12. Spleen cells were cultured ex vivo without further stimulation for 24 hr and culture supernatants tested for (C) TNF α and (D) IL-10 levels by ELISA (N = 5/group of lupus mice and 2 for BALB/c controls). ** $p < .01$; *** $p < .001$; **** $p < .0001$ PAM3 vs PBS treated NZB/W mice.

3.4.3 Effect of PAM3 on kidney pathology and survival

As the disease progresses in the NZB/W mice kidney damage occurs. Clinically the appearance of significant amount of protein in urine is one of the earliest signs of almost all renal diseases [278]. The level of proteinuria was reduced in both early and late treatment groups at 6 mos of age ($p < 0.005$ in the early and < 0.01 in the late treatment groups, Figure 3.19 A). Blood urea nitrogen (BUN) and creatinine levels are primarily used to evaluate kidney function for the diagnosis of kidney disease. Urea is an end product of metabolism and is filtered from the blood through the

kidneys. Increased BUN levels are associated with kidney disease or failure. Creatinine is a breakdown product in the muscles like urea it is filtered by the kidneys in healthy conditions. Kidney damage was confirmed by the increased serum BUN and creatinine levels from mice that are 6 mos of age. The serum BUN and creatinine levels were reduced to normal BALB/c control levels, in both early and late treatment groups at 6 mos of age. The ratio of BUN: Creatinine is reported to be more reliable than BUN levels alone because the BUN can be affected by dietary and physiologic conditions. Results showed that BUN: Creatinine ratio was significantly dropped in both early and late treatments (Figure 3.19 B).

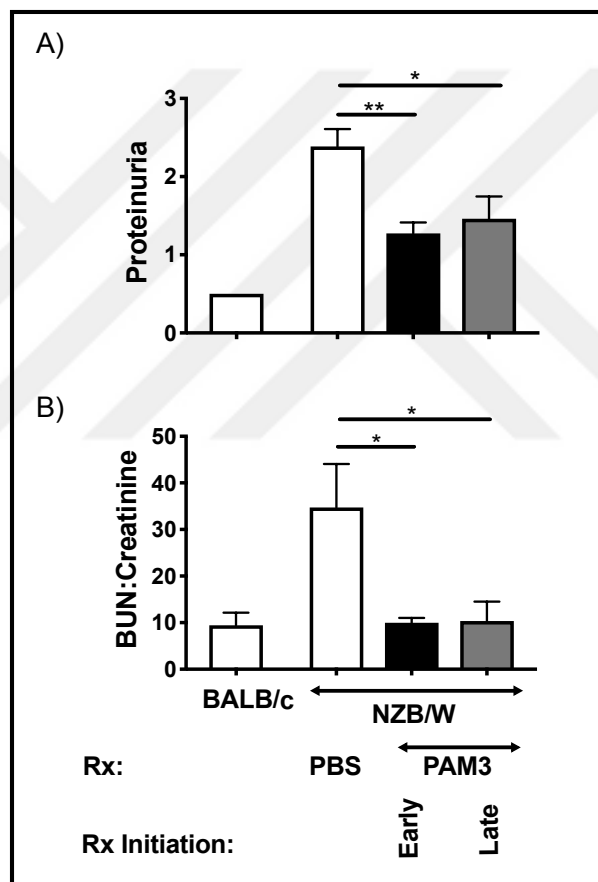


Figure 3.19: Effect of PAM3 treatment on lupus nephritis.

NZB/W mice were treated with PAM3 or PBS as described in Fig 4. A) Proteinuria was measured at 6 mos (N=13 for each PAM3 treated group and 22 for PBS treated mice). Serum levels of B) BUN: Creatinine was calculated from mice that are 6 mos of age (N=5 for NZB/W, N=3 BALB/c). * p<0.05; ** p<0.01 PAM3 vs PBS treated NZB/W mice.

The natural history of disease was examined by monitoring kidney histology. Immunohistochemical staining of kidneys from animals starting at 6 months of age

showed that number of CD206⁺ M2 macrophages remained low as disease progressed in the PBS group. By comparison, the number of M2 macrophages in the kidneys of PAM3 treated mice was higher and increased over time, consistent with the normalization of M1:M2 ratio observed in Figure 3.17 (Figure 3.20 A, B). Kidney histology was evaluated by PAS and H&E staining. Glomerulonephritis was scored based on interstitial inflammation (caused by infiltrating leukocytes), hyaline deposits (immune complex deposits) and crescent formation (highly proliferative cells) (Figure 3.20 C-G). Histological analysis of these kidneys confirmed that glomerular and tubular infiltration by inflammatory cells was delayed by PAM3 treatment. At 6 months of age, the glomerulonephritis score of PAM3 group was 3.2 vs 5.7 in the PBS group. By 11 months the PAM3 score was 5.0 while in the PBS group it had risen to 7.2 (Figure 3.20 C-G).

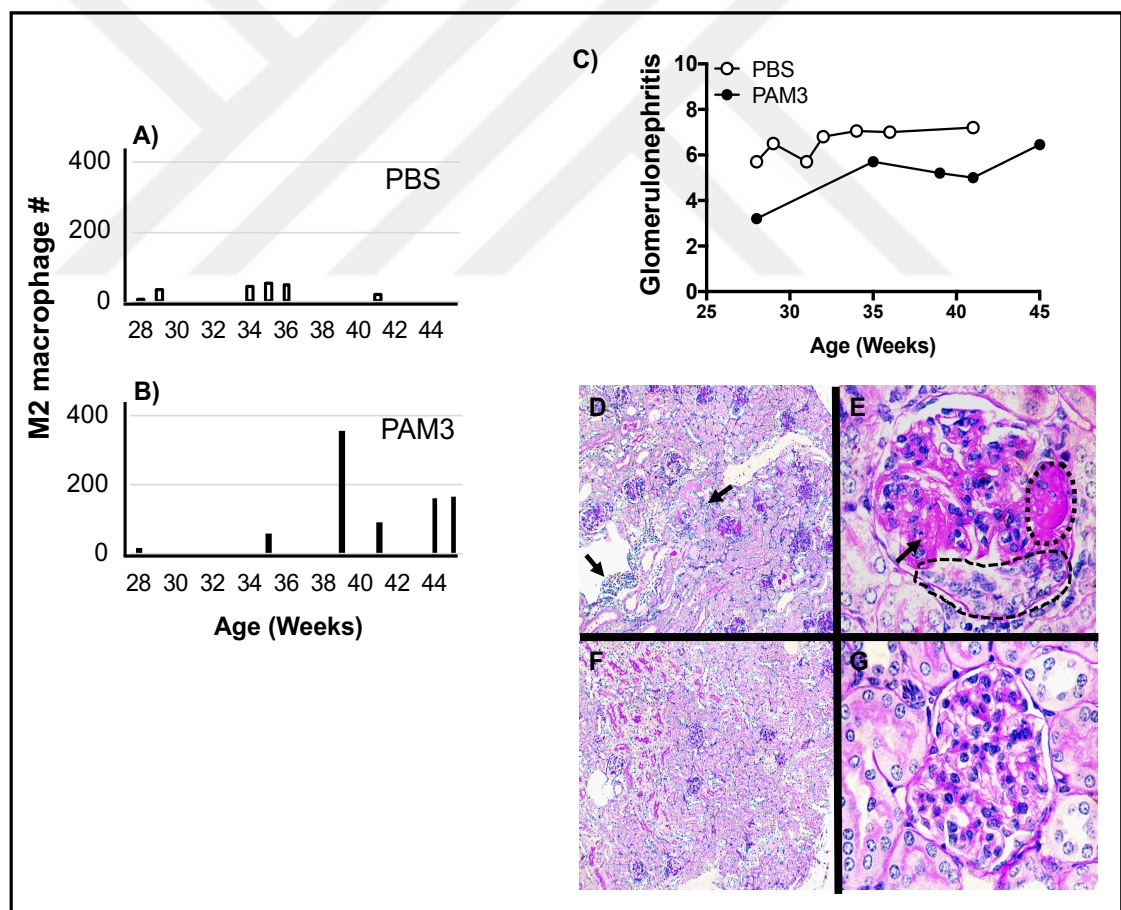


Figure 3.20: Effect of PAM3 treatment on kidney pathology and the accumulation of M2 macrophages.

NZB/W mice were treated as described in Fig 6. A, B) The number of M2 macrophages present in their kidneys was monitored over time by immunohistochemical staining of PBS and PAM3 treated mice. C) The severity of

glomerulonephritis was evaluated histologically. N=9 for PBS and 5 for PAM3 treated animals. D-G) Representative PAS stained kidney sections from 28-week-old NZB/W mice treated with PBS (D, E) or PAM3 (F, G). Note the increased interstitial inflammation (arrows) in panel D and hyaline deposits (--), cellular crescent formation (—) and karyorrhexis (arrow) in panel E vs the slight increase in endocapillary proliferation in panel G. Panels D, F were 100x and panels E, G were 800x magnification.

Survival of mice that received PAM3 was monitored until mice were 45 weeks old. At 40 weeks of age, 60% of mice in the early PAM3 treatment group had survived compared to only 5% in the PBS group (Figure 3.21, $p=.0037$). Initiating PAM3 treatment at 13 rather than 7 weeks of age was not as effective at slowing disease and prolonging survival.

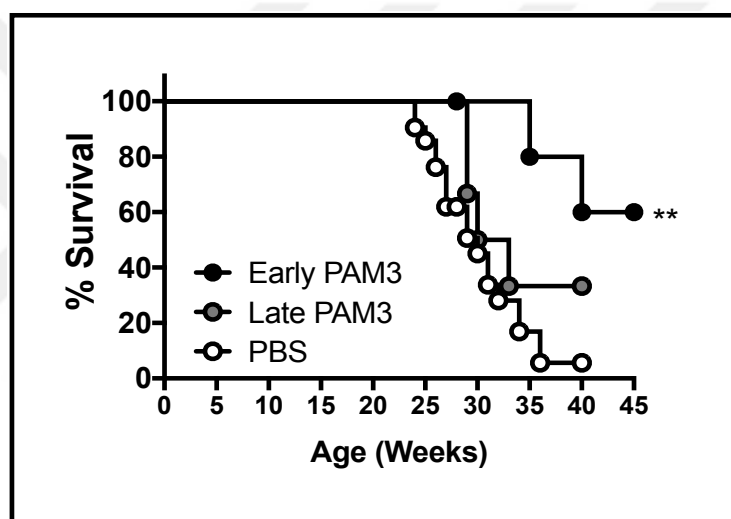


Figure 3.21 Survival was monitored through 45 weeks of age in mice treated with PAM3 at 7 or 13 weeks of age (N=6/group) or PBS treated controls (N=21).

** $p=.0037$ when compared to PBS group, Log rank test.

3.5. Encapsulation of different CpG types into liposomes enhance their efficacy as vaccine adjuvants

Previous studies demonstrated that D- and K-type CpG ODNs induce different immune responses [63, 279-283]. Although both are strictly dependent on TLR9, K-type CpG ODN trigger pDCs to mature and secrete $\text{TNF}\alpha$ while D-type CpG ODN stimulate pDC to produce $\text{IFN}\alpha$. It was reported that liposomal encapsulation increases immunostimulatory effects of CpG ODN's [72, 78]. So, I investigated if combining liposomal D-and K-type of CpG ODNs would enhance their efficacy as tumor vaccine adjuvants.

3.5.1. Combinations of liposomal CpG ODNs enhanced production of both IFN α and TNF α from human PBMC and altered APC function

According to the literature when both types of CpG ODNs are introduced simultaneously to human pDC in their free forms, only K-type CpG specific response is observed and D-type CpG response is blocked [284]. Combining neutral liposomal D- type CpG (from now on will be referred as; NL(D)) with cationic liposomal K-type CpG (will be referred as; CL(K)) significantly increased production of both cytokines. Our results showed that the combination NL(D)+ CL(K) significantly increased IFN α secretion from human PBMC whereas upon stimulation with free CpG ODNs (D+K) IFN α secretion was decreased greatly (Figure 3.22 A). Moreover, the frequency of TNF α ⁺ cells were elevated upon stimulation with the liposomal combination when compared to their free forms (Figure 3.22 B). Improved APC activity/ function was confirmed by increased frequency of CD80 and CD86 double positive cells with liposomal combination (Figure 3.22 C).

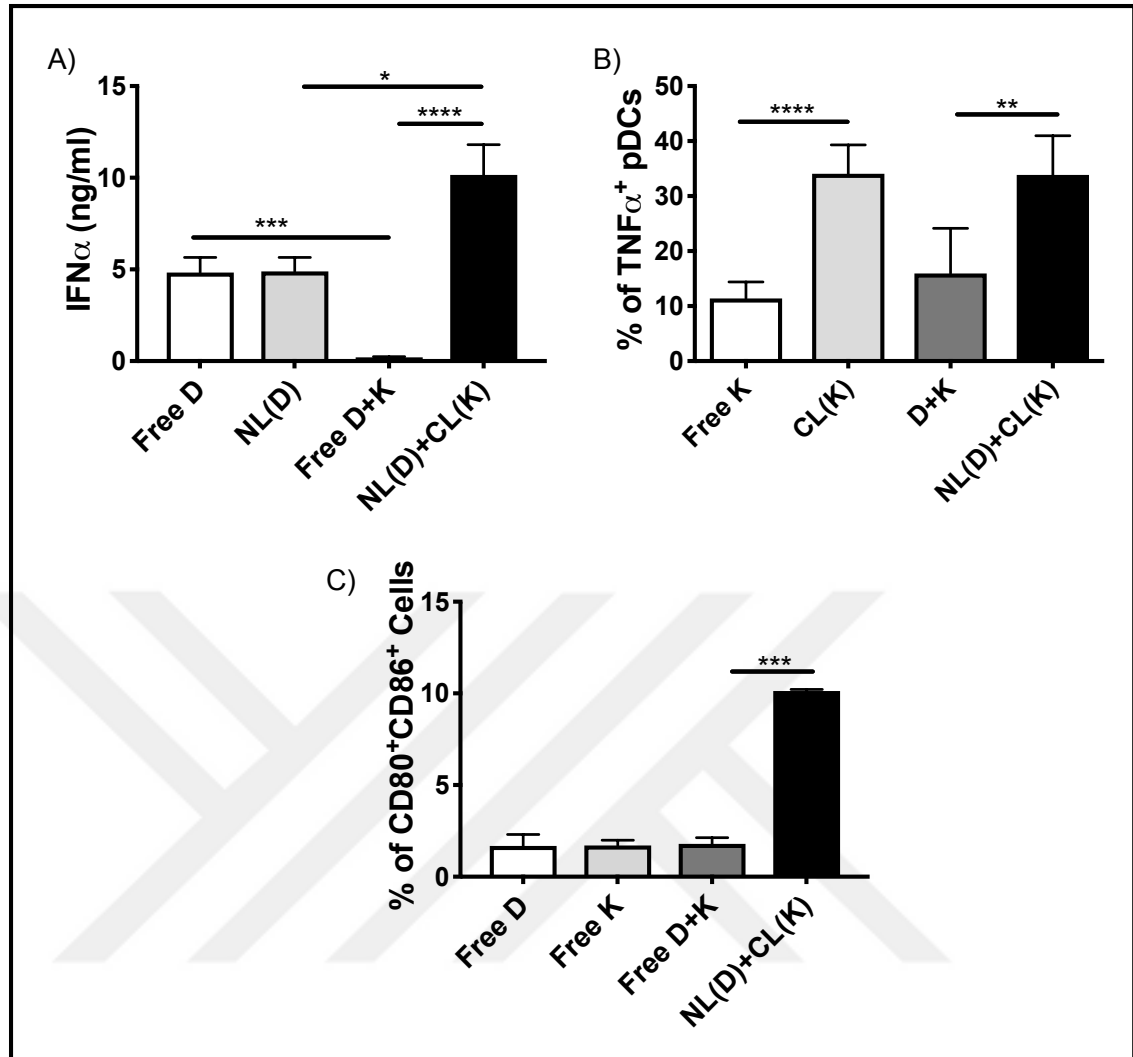


Figure 3.22: Combining liposomal CpGs overcame the dichotomy and altered the immune response from human PBMC.

IFN α (A) secretion was detected by cytokine ELISA from human PBMC culture supernatants whereas TNF α (B) was detected by intracytoplasmic staining of pDCs gated by BDCA2 and CD123 (N=4). Altered function of APCs were confirmed by flow analysis of C) CD80+CD86+ double positive cells from human PBMC (N=2) upon stimulation with indicated groups. * $p < .05$ ** $p < .01$; *** $p < .001$; **** $p < .0001$

3.5.2. The sub-cellular localization of either D-or K-type CpG ODN did not change upon liposomal encapsulation

Increased IFN α secretion from human PBMC emphasized that liposomal formulation of both CpG achieved to overcome the suppression caused by K-type on the D-type specific response (which was observed upon free CpG stimulation; D+K) and induce a synergistic immune response by using both CpG ODN in liposomal forms (Figure 3.22 A, B). However, the underlying mechanism that leads to this

alteration is unclear. It has been established that D-type CpG ODN localizes and retains in the early liposome whereas, K- type CpG ODN passes to the late endosome rapidly [63]. Thus, we investigated whether the additive effect we observe by combining the liposomal formulations, was due to an altered sub-cellular localization of either CpG ODN. According to confocal microscopy studies, liposomal D-type CpG accumulated to the early endosome confirmed by the co-localization of labeled D-type CpG encapsulated within neutral liposome with Transferrin (TF) but not with late endosome; LysoTracker (LY) in support of previous reports (Figure 3.23 A, B). On the other hand, K-type CpG ODN encapsulated within cationic liposomal localized within late endosome (LT) but not in early endosome (TF) again consistent with previous studies (Figure 3.23 C, D). Incubation of liposomal combination showed that, simultaneous stimulation of NL(D) with CK(L) did not changed the localization of either CpG ODN (Figure 3.23 D-G). Confocal results showed that neither liposomal encapsulation or the combination of both CpG ODN in liposomes, affect the sub-cellular localization of CpG ODN.

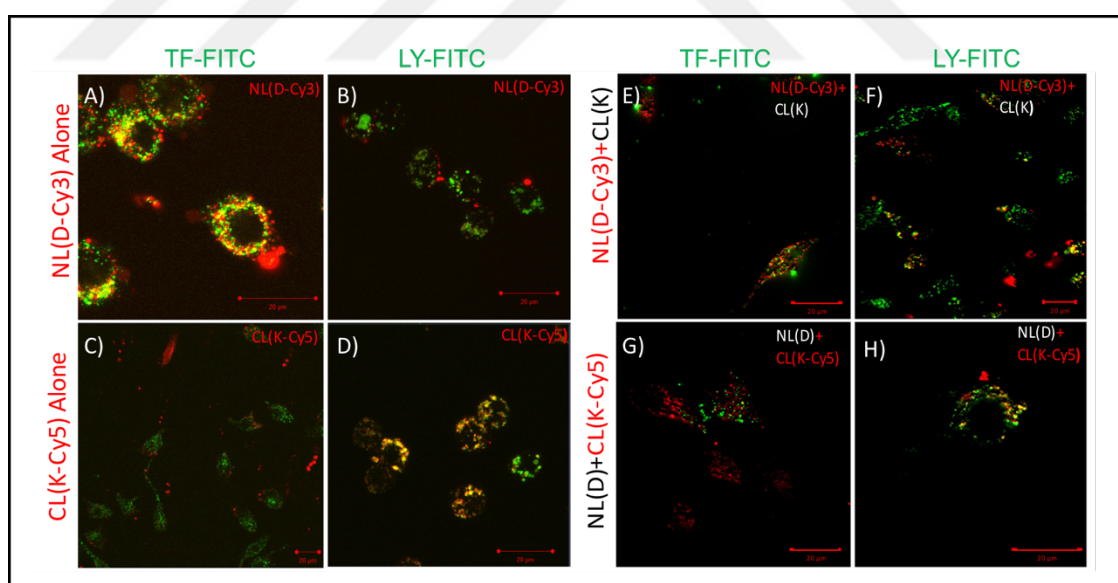


Figure 3.23: The sub-cellular localizations of D-and K-type CpG ODN did not change upon encapsulation within liposomes.

Early and late endosomes were stained with Transferrin-FITC and LysoTracker-FITC respectively. Cells were incubated with either alone liposomal Cy5 or Cy3 labeled CpG ODNs (A-D) or in combination (E-H) of labeled and unlabeled CpG ODN containing liposomes. Images were taken with LSM 710 confocal microscope at 63x magnification.

3.5.3. Cationic liposome encapsulation of K-type CpG ODN delayed its internalization by pDCs, allowing for D-type CpG ODN to be internalized and induce the secretion of IFN α

It is possible that the dichotomy caused by K-type CpG ODN, is due to a competitive activation of TLR9 between CpG ODNs. Since D-type CpG ODN forms quadruplex structures, it is a bigger compound in size than K-type CpG, which might be slowing down its internalization, whereas K-type CpG ODN quickly accumulates to late endosome and activates NF κ B signaling pathway, thus blocking the activation of D-type specific responses. As, liposomal combinations of both CpG ODN's achieved to rescue D specific response, we investigated the internalization kinetics of encapsulated CpG ODNs. Encapsulation of K-type CpG ODN into cationic liposome delayed its internalization by 6-fold into pDC both in combination or alone (Figure 3.24 A). Whereas, at 6 hr neutral liposome encapsulation of D-type CpG ODN increased its internalization by 3-fold both in combination and alone (Figure 3.24 B).

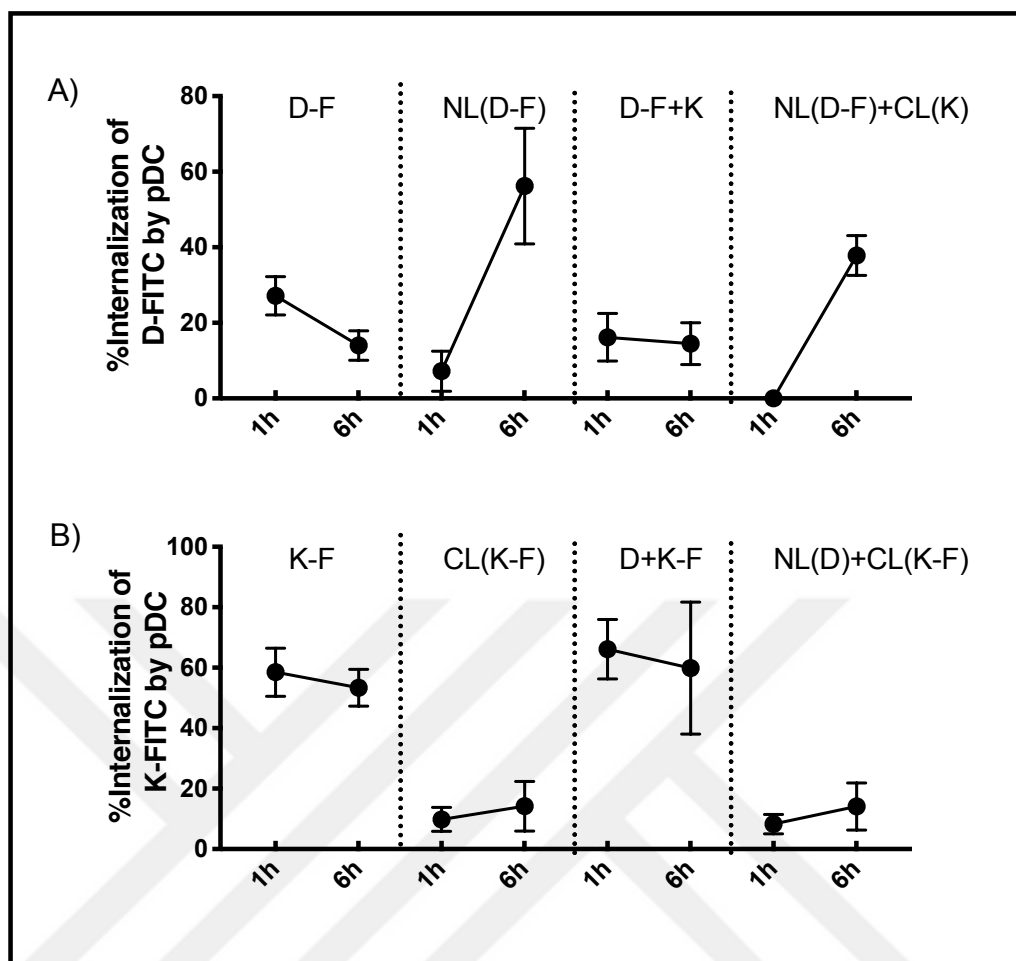


Figure 3.24: Liposomal encapsulation changed the internalization rates of CpGs.

FITC labeled liposomal or free K-type (A) and D-type (A) CpG ODNs were incubated with human PBMCs for 1 hr and 6 hr, samples were analyzed by flow cytometry and pDCs were gated by CD123 and BDCA-2 expressions (N=4). Internalization of CpGs was calculated by subtracting binding (incubation @ 4 C) from the uptake (incubation @ 37 C) values.

3.5.4. Liposomal combination induced an altered immune response in mice

Mouse cells differ from humans in terms of TLR expressions. In mice, addition to pDC and B cells, mouse macrophages also express TLR 9 [285]. We first investigated whether our combination was achieving synergistic activation in mouse as well, to confirm that mouse can be used as a model organism for further investigating the improved adjuvant properties of our combination. Splenocytes of C57BL6 mice were stimulated with the combination (NL(D) +CL(K)) that was found to be the most effective formulation in human. We measured secretion of IL6, IL12 and IFN γ with cytokine ELISA 24 hr later from supernatants of mouse

splenocytes (Figure 3.25 A-C). Our results showed that combination ‘NL(D)+CL(K)’ achieved an altered immune activation like in human, observed by significantly increased cytokine production when compared to D+K and their alone liposomal forms.

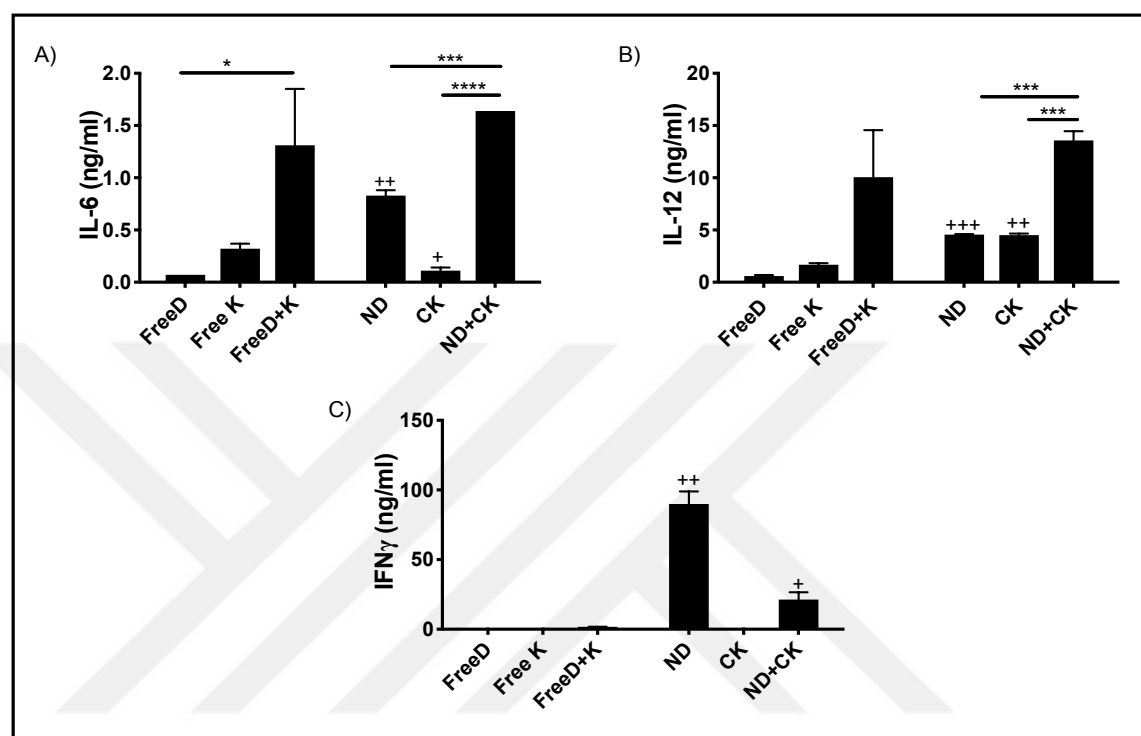


Figure 3.25: Liposomal combination altered immune response from mice splenocytes.

Secretion of IL-6 (A), IL-12(B) and IFN γ (C) were measure by cytokine ELISA from splenocytes cultures after 24 hr of stimulation. * $p < .05$ ** $p < .01$; *** $p < .001$; **** $p < .0001$ compared to indicated groups whereas ++ $p < .01$; +++ $p < .001$ compared to free forms.

3.5.5. Liposomal combinations conferred a Th1 biased anti-OVA immunity in vivo and blocked formation of tumors

We assessed that liposomal combinations induced a synergistic immune response in vitro, so next we set up an in vivo vaccination experiment to test their efficacy of the formulation as a vaccine adjuvant. Since, alone forms of either liposomal CpG ODN's was not as effective as combinations in vitro studies, it was not worth adding them to the in vivo setup. C57BL/6 mice were immunized intraperitoneally with indicated groups (Figure 3.26 A) on the days 0 and 14. OVA-Specific IgG responses were determined 2 weeks after the booster injections according to the immunization

schedule (Figure 3.26 A). IgG1 and IgG2c were analyzed since the subtypes depend on the type of T-cell activation (Th2 vs Th1, respectively). Sera of individual mice were serially diluted and OD versus reciprocal serial dilution curves were generated (Fig 3.26 B). IgG2c responses were significantly higher in liposomal combinations compared to free forms of D+K, whereas elevated levels of IgG1 were observed with the free forms of CpG ODNs and OVA alone. The results suggested a strong Th1 biased immunity caused with all the liposomal combinations.

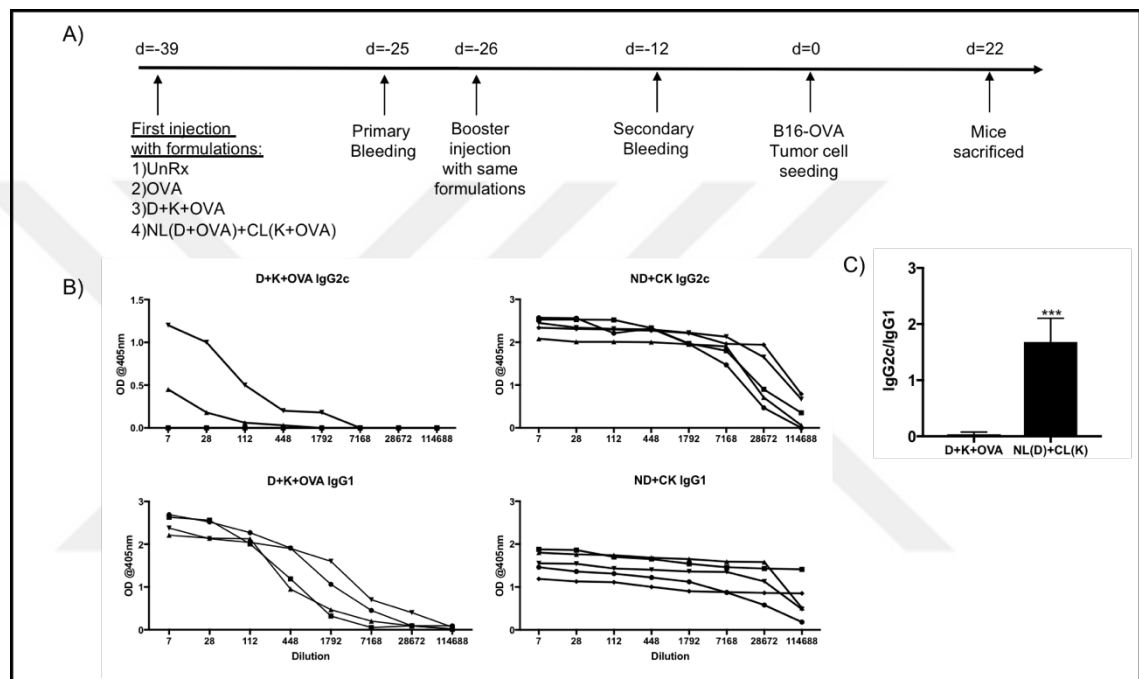


Figure 3.26: Liposomal combination induced a strong Th1 biased immunity noted by the IgG2c/ IgG1 ratios.

Mice received liposomal formulations that includes OVA as the antigen on days indicated at the timeline (A) and bled twice after 15 days of injection. The anti-OVA IgG2c and IgG1 levels were measured after the secondary bleeding by ELISA from sera that were serially diluted (B), the IgG2c/IgG1 ratios were calculated from 7168x diluted sera (C). (N=5 mice/group) * $p < .05$ ** $p < .01$; *** $p < .001$; **** $p < .0001$

To assess the OVA-specific cell-mediated immunity will protect mice from tumor formation, mice were challenged sub-cutaneously on day 0, (12 days after the secondary bleeding) with OVA expressing melanoma cell line B16 (Figure 3.26 A). Tumor volumes were measured every two days for 14 days. Mice that were vaccinated with liposomal combinations showed latency in tumor development and 40% were tumor free even at day 22 post tumor cell inoculation (Figure 3.27 B). Our results indicate that liposomal combinations triggered an enhanced anti-tumor

response compared to combination of free CpG ODNs thus leading to a protection from tumor formation.

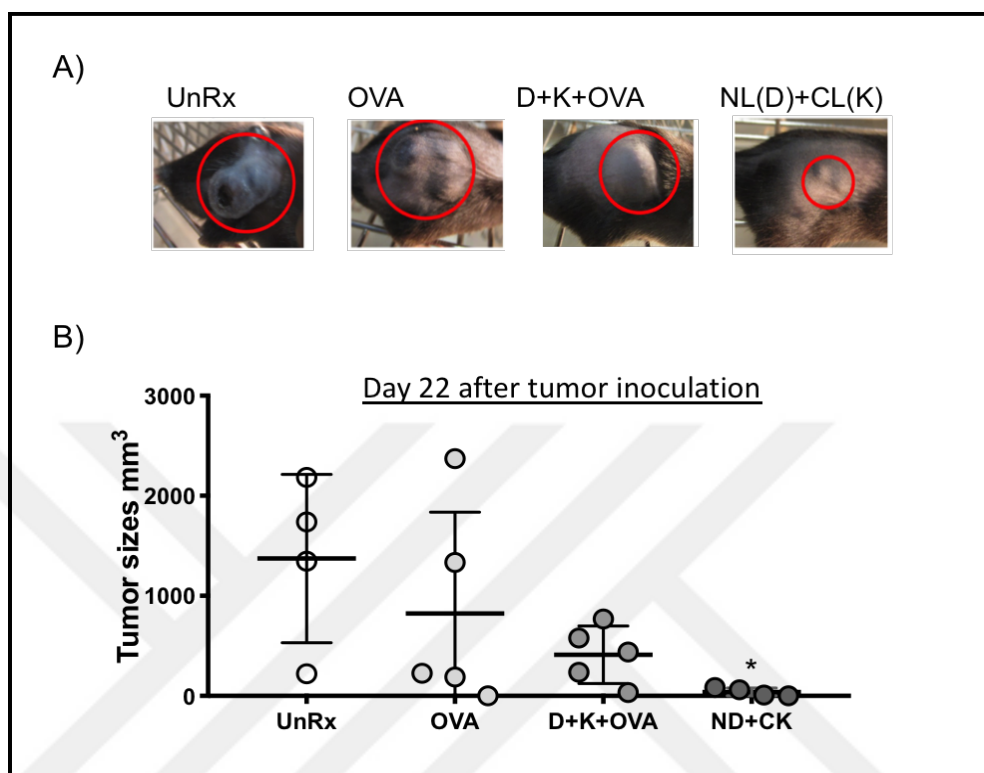


Figure 3.27: Liposomal combination blocked tumor formation.

Representative photos were taken (A) and tumor sizes (B) were measured at day 22 after tumor inoculation by caliper (N=4-5/ group).

Chapter 4

Discussion

Monocytes are derived from myeloid progenitors and represent 5-10% of peripheral leukocytes in humans. They are key players in immune defense, inflammation and homeostasis. They clear pathogens, remove dead cells, and contribute to the development of adaptive immune responses [286]. Monocytes can be stimulated to differentiate into either macrophages or dendritic cells, each of which plays a role in protecting the host from infection.

Monocytes have been classified into 3 main subsets based on surface marker expression and functional activity. Monocytes that express CD14 but not CD16 are classified as ‘classical’ monocytes. These are phagocytic but do not have inflammatory attributes. On the other hand, CD16 expressing monocytes with low expression of CD14 are termed ‘non-classical’ monocytes which are inflammatory. Monocytes expressing both CD14 and CD16 are termed ‘intermediate’ monocytes and represent a transitional subset with both phagocytic and inflammatory features [86]

Abnormalities in monocyte phenotype and function reportedly contribute to several autoimmune disorders [112, 287, 288]. The findings described above show that the monocyte percentages in PBMC were significantly increased in vasculitis patients, but not in lupus or myositis patients. The latter finding is consistent with previous reports on monocytes from those populations [156, 252] (Figure 3.1 A, Figure 3.6 A and Figure 3.11 A).

My studies indicate that a shared characteristic of the patients with autoimmune diseases studied in this thesis is a significant increase in monocytes of the inflammatory subtypes coupled with a decrease in non-inflammatory classical monocytes (Figure 3.1 B, Figure 3.6 B and Figure 3.11 B). In lupus, the relative frequency of these monocyte subpopulations did not differ between patients with active (SLEDAI>6) vs inactive disease (Figure 3.1C, D). By comparison, the

percentage of inflammatory monocytes was significantly increased in adult myositis patients (AM) vs those with juvenile myositis (JM) (Figure 3.13 A, B). Although the clinical presentations of JM and AM are similar, disease progression in AM patients tends to be more severe and can lead to cancer or lung disease whereas in JM patients long term survival and prognosis is better [215, 289]. The increased frequency of inflammatory monocytes suggests either that such cell contribute to disease progression or are generated as a consequence of the disease. This increased in inflammatory monocytes was previously reported in patients with lupus and vasculitis but not myositis [156, 252]. While the behavior of each monocyte subtype after stimulation would be of interest, limitations in sample volumes (particularly of the non-classical components) prevented such studies. Instead, I analyzed the response of monocytes to various stimuli in an effort to monitor their ability to differentiate into macrophages with distinct phenotypes and functions.

These macrophages range from M1-like inflammatory to M2-like immunosuppressive macrophages. Different macrophages subtypes have the ability to either induce or suppress immune responses, by producing cytokines or interacting with other immune cells. An altered balance between M1 and M2 macrophages has been observed in many disease states. Cancer is characterized by an over-abundance of immunosuppressive M2-like macrophages, yet studies indicate that patients would benefit from an increase in anti-tumor M1-like macrophages [286]. Wang et al showed that peripheral blood monocytes from cancer patients were prone to differentiate into M2-like macrophages in the absence of further stimulation. This suggests that tumor-derived factors supported the generation of macrophages that block rather than support anti-tumor immunity [130].

In contrast, autoimmune diseases are generally characterized by an increased frequency of M1-like macrophages that support the development of inflammation. Recent studies suggest that the M1:M2 macrophage balance can influence disease progression in lupus, as adoptive transfer of M2 macrophages in a murine model reduced disease severity [251]. In giant cell vasculitis, M1-like macrophages contribute to the formation of large granulomas by fusing with T cells. Moreover, secretion of inflammatory cytokines and reactive oxygen species by M1-like macrophages can cause vascular inflammation [252-256]. In muscle biopsies from

myositis patients, infiltration of macrophages with characteristic features of M1-like macrophages was observed which would be consistent with their having a role in the pathogenesis of disease [258, 259]. An imbalance in M1:M2 ratio in these disease states is likely driven by increased levels of pro-inflammatory cytokines such as IL-12, TNF α , IFN γ and type I IFNs [236, 238, 240]. These support the generation of M1-like macrophages via pathways involving JAK/Stat-1, NF- κ B and interferon regulatory factors. Interestingly, my research shows that monocytes from lupus, vasculitis and myositis patients are predisposed to differentiate into M1-like macrophages when not further stimulated *in vitro*. I postulate that this reflects their *in vivo* exposure to factors that support the preferential generation of inflammatory macrophages (Figure 3.2 C, Figure 3.7 B, Figure 3.12 B). These findings support the conclusion that an imbalance in the M1:M2 ratio may contribute to the pathogenesis of multiple autoimmune diseases. Hence, increasing the frequency of immunosuppressive M2-like macrophages could represent a useful strategy for the treatment of autoimmunity. Previous studies report that human monocytes differentiate into M1-like macrophages upon stimulation with most of the TLR agonists. The only exception to this pattern was the TLR2/1 agonist PAM3 which promoted the generation of M2-like macrophages from human monocytes [131].

As monocytes from patients with any of these diseases behaved similarly in the absence of *in vitro* stimulation, I sought to identify shared factors that might affect their maturation into M1 macrophages *ex vivo*. First of all, each population of patient donor had increased frequencies of inflammatory monocytes that are more likely to differentiate into inflammatory macrophages [86]. However, this cannot be the sole explanation because the frequency of inflammatory monocytes only accounted for 15% of the total monocyte pool. Other parameters that are likely to contribute to M1 biased macrophage differentiation could include environmental and/or genetic factors. As mentioned before all these patients had increased levels of inflammatory cytokines that contribute to a skewed M1:M2 ratio *in vivo*, which is most probably one of the reasons why their monocytes are more prone to differentiate into M1 macrophages *in vitro* as well. It was believed that monocytes are a homogenous population that could differentiate into a homogenous population of macrophages or DCs upon encountering stimuli. With the development of single cell RNA sequencing (scRNA-seq) technology, it is now believed that monocyte

populations are heterogeneous, although whether this heterogeneity depends on environmental factors is still being investigated. Studies suggest that pre-committed monocytes might exist. It was recently shown by a combination of multicolor flow cytometry and single cell qPCR that monocyte populations in murine bone marrow are already predetermined to become inflammatory macrophages [290]. Recent advancements in the field of scRNA-seq greatly advanced our understanding of the population structure of monocytes/macrophages in diseases such as cancer, atherosclerosis and Alzheimer's, thus it would be compelling to apply this technology for autoimmune disease in order to understand the behavior of their monocytes. Additionally, gene expression studies in lupus patients revealed that during monocyte to macrophage differentiation dramatic changes in gene expression profiles were observed including genes important to lipid and carbohydrate metabolism. Expression of these genes could alter energy metabolism and mitochondrial function during cellular differentiation, thereby programming monocytes to differentiate into macrophages with inflammatory attributes. Moreover, it was reported that specific signal transduction genes were downregulated during macrophage differentiation in SLE patient monocytes. These genes included JAK2 and STAT6 which help mediate M2 macrophage differentiation [291]. It would be interesting to see whether similar gene expression differences are observed in vasculitis and myositis patient monocytes. A recent study also reported that IFN γ downregulates genes associated with M2 macrophage differentiation, thus programming monocytes to become M1 macrophages. Of note, in the presence of stimuli that induce M2 macrophages, this effect was replaced by the upregulation of genes associated with M2 macrophages. My studies showed that patient monocytes respond identically to those from normal volunteers when stimulated with PAM3, R848, IFN γ or MCSF. Since increased levels of IFN γ are found in all three disease states, another reason their monocytes may be prone to differentiate into M1 macrophages could be their exposure to elevated levels of IFN γ [292].

Current therapies for SLE include the use of immunosuppressive drugs, steroids and agents that deplete/inhibit autoreactive B cells [159, 293, 294]. Novel treatments that target the interferon pathway and B/T cell signaling are being evaluated in phase III trials. The first line treatment for large vessel vasculitis or dermatomyositis are

corticosteroids started as early as possible. Unfortunately, over 80% of vasculitis patients report experiencing treatment-related side effects and disease in some individuals is corticosteroid resistant [295]. Recent studies support additional therapeutic options for the treatment of vasculitis: IL-6 receptor blockers, T cell checkpoint inhibitors, antibodies against IL-12, and agents that target Th1 and Th17 cell generation are all of interest in this regard [296]. For treatment of dermatomyositis, immunosuppressive therapies can be started along with the corticosteroids. Although there is no convincing evidence for the efficacy of immunosuppressive drugs in that disease, an international consensus of experts has recommended they should be used [297]. IVIG is an alternative when toxicity caused by immunosuppressive agents becomes severe [298]. Recent studies suggest that depletion of B cells by monoclonal antibodies has potential in the treatment of myositis [299].

The patients evaluated in this study were treated with the conventional agents described above. Lupus, vasculitis and myositis patients received hydroxychloroquine, prednisone, NSAIDs and immunosuppressive drugs including mycophenolate mofetil (MMF) and azathioprine (AZA) (Tables 1-3). Hydroxychloroquine is an antimalarial that also interferes with TLR7 and TLR9 signaling [300, 301]; prednisone is a glucocorticoid that inhibits T and B cell responses [302]; MMF is an immunosuppressive agent that inhibits lymphocyte proliferation and antibody production [303]; while AZA regulates DNA synthesis thereby inhibiting cell proliferation [159]. None of these drugs is known to selectively act on monocytes/macrophages or influence monocyte differentiation. Indeed, the response of patients' monocytes did not vary as a function of what drugs they were using (data not shown).

My thesis focused on characterizing the macrophages generated by PAM3 treatment of monocytes from patients with autoimmune disease. Both phenotypic and functional analyses were performed to clarify their behavior. I also tested the therapeutic activity of PAM3 in a murine model of lupus to confirm predictions from results of human clinical samples.

FACS purified monocytes were incubated for 5 days with macrophage colony stimulating factor (M-CSF, a positive control for the generation of M2-like

macrophages) or IFN γ /R848 for the generation of M1-like macrophages. The concentration of the agents used was based on optimization studies previously performed in the lab [130, 131]. Macrophages were detected by their expression of the 25F9 surface marker that is uniquely expressed by mature human macrophage [264]. CD163, a scavenger receptor expressed preferentially by M2-like macrophage, was used to differentiate between M1- and M2-like macrophages. As expected, MCSF and PAM3 induced the generation of M2-like macrophages whereas IFN γ and R848 generated M1-like macrophages (Figure 3.2, Figure 3.7, Figure 3.12). The behavior of monocytes from healthy controls was nearly identical to that of patient monocytes in terms of their response to PAM3, MCSF, R848 and IFN γ .

While MCSF can induce monocytes to differentiate into M2-like macrophages, it has additional effects on other cell types that reduce its clinical utility. MCSF stimulates both dendritic cells and monocytes to proliferate [82, 271-273]. An overabundance of such cells can worsen lupus, as was found experimentally when the mouse homologue CSF-1 was administered to MRL/lpr mice [241]. Thus, MCSF was not included in the functional studies of generated macrophages.

Endocytosis is a characteristic feature of M2-like macrophages. A zymosan uptake assay was performed to evaluate the endocytic ability of macrophages generated by PAM3 from healthy control and patient samples. PAM3 generated macrophages with high endocytic ability whereas M1 stimuli did not (Figure 3.4 B, Figure 3.9 A, Figure 3.15 A). The endocytic ability of PAM3 generated macrophages also correlated with the expression of the scavenger receptor CD163 (Figure 3.4 B, Figure 3.9 B, Figure 3.15 B). CD44, a marker associated with the clearance of apoptotic cells, was significantly elevated in monocytes stimulated with PAM3, consistent with their increased zymosan uptake ability (Figure 3.3 B, Figure 3.8 B, Figure 3.14 B). Another marker which is associated with endocytic abilities is CD14. Upon PAM3 stimulation both healthy control and lupus monocytes had significantly altered CD14 expression (Figure 3.3 A, Figure 3.8 A, Figure 3.14 A).

In lupus patients, impaired clearance of apoptotic cells has been associated with increased autoantibody production. Apoptotic cells do not activate an immune

response unless they are phagocytized. Rather, they leak DAMPS and self-antigens in a process known as 'secondary necrosis'. Necrotic cell components induce the secretion of cytokines by macrophages which in turn cause B cell stimulation [275]. When macrophages needed to remove apoptotic cells aren't present in sufficient numbers, the production of autoAbs and further inflammation results. The zymosan uptake assay is a marker for the ability of PAM3 generated macrophages to clear apoptotic cells and thus reduce the likelihood of continued inflammation. This conclusion was supported by *in vivo* studies of lupus prone NZB/W mice in which PAM3 treatment significantly reduced IgG anti-dsDNA autoantibody production (Figure 3.18 A). Although impaired clearance of apoptotic cells is not reported in vasculitis or myositis patients, increased autoantibody production is found in both diseases [304, 305]. It would be interesting to develop animal models to investigate whether the accumulation of apoptotic cells resulting from impaired clearance by macrophages might be responsible for autoantibody production in vasculitis and myositis as well.

I also investigated a marker was associated with antibody production and reported to be increased on SLE and vasculitis patient macrophages. CD40 is the receptor for CD40L and is found on B cells, T cells and macrophages. If the interaction between CD40 and its ligand is excessive, autoantibody production can result. Consistent with previous reports, I found that CD40 expression was significantly elevated in freshly isolated monocytes from lupus patients than healthy controls [232]. This was also the case for monocytes from vasculitis patients, and these cells matured into M1-like macrophages without further stimulation in culture. In vasculitis patients it was shown that interaction of CD154 (a ligand for CD40) with CD40, contributes to granulomatous inflammation [306]. CD40 expression was significantly elevated on the macrophages generated by M1 stimuli but not those generated by PAM3 (Figure 3.4 C, Figure 3.9 C, Figure 3.15 C). Increased expression of markers such as CD14, CD44 and CD163 observed in the zymosan uptake experiments indicate that PAM3-generated macrophages have high endocytic abilities consistent with their M2 macrophage phenotype. Such functionality would be of benefit in the treatment of lupus for the efficient clearance of apoptotic cells. Moreover, lack of CD40 expression implies that PAM3-generated macrophages will not contribute to any further activation of lymphocytes.

Another important feature of M2-like macrophages is their ability to suppress T cell activation and proliferation[307]. Bayik et al reported that the macrophages generated by treating monocytes from normal human donors with PAM3 suppressed T cell proliferation and activation [131]. The same study could not be performed on patient samples because it required an additional sample collection (due to differences in how long it takes to mature macrophages vs T cells). Instead, changes in surface expression of CD273 was investigated. CD273 on macrophages interacts with PDL-1 on T cells, resulting in their inhibition [308]. Although M2-like macrophages are reportedly to primarily express this marker, macrophages generated by PAM3 did not up-regulate its expression in a statistically significant manner (although an increase was seen after MCSF stimulation) (Figure 3.4 D, Figure 3.9 D, Figure 3.15 D). It is likely that the suppression of T cell proliferation by PAM3-treated monocytes does not depend solely on the expression of CD273. For example, the activation of STAT6-dependent signaling pathways in M2 macrophages has a suppressive effect on T cells [307]. Another mechanism by which M2 macrophages can inhibit T cell proliferation is via metabolic starvation. T cells require arginine for proliferation and arginine is depleted due the increased expression of Arg1 by M2 macrophages [309, 310]. Thus, efforts to fully understand the mechanism by which PAM3-generated macrophages suppress T cell proliferation will require additional experiments. On the other hand, I observed that IFN γ stimulated monocytes from both control and patient samples showed significantly elevated CD273 expression, this increased expression can be explained by a negative feedback loop. IFN γ is classically considered to be a pro-inflammatory cytokine. However, in chronic inflammatory conditions, IFN γ can mediate regulatory functions that limit inflammation-induced tissue damage [311-316]. In this context, IFN γ can induce the differentiation of Treg cells that suppress Th1 cells or promote the differentiation of MDSC. Moreover, it was reported that IFN γ can induce PDL-1 expression by macrophages that suppress T cell activation through a negative feedback loop, thereby minimizing the negative effects of IFN γ [311, 317-320]

The ability of M1-like macrophage to secrete cytokines was used to assess their functional capability. The production of pro-inflammatory cytokines such as TNF α

and IL12 was significantly increased in macrophages generated by M1 stimuli. These cytokines have been described as worsening disease in lupus and vasculitis. IL-12 levels are significantly elevated in GCA lesions of vasculitis patients and TNF α levels in the serum of lupus and vasculitis patients correlates with disease activity [321]. Similarly, IL-12 and TNF α are also overexpressed in the blood and muscle biopsies of patients with myositis [322].

My studies of NZB/W mice found that the frequency of M1 macrophages was abnormally increased, consistent with what has been reported in lupus patients [277]. This was accompanied by increased levels of autoantibodies and the inflammatory cytokines IL-12 and TNF α again similar to human lupus. Although neutralizing IL-12 was shown to prevent the development of Th1-mediated autoimmune disease, anti-TNF α was not effective for the treatment of vasculitis [323]. I found that the production of IL-12 and TNF α was significantly reduced when NZB/W mice were treated with PAM3 (Figure 3.18 B, C). Secretion of the pro-inflammatory cytokines IL-12 and TNF α is a classic feature of M1 macrophages. Thus, a reduction in the secretion of those cytokines, along with a decreased M1:M2 ratio, is likely to be associated with decreased M1 macrophage frequencies following PAM3 treatment. It was consistent with my observation that human macrophages generated by PAM3 stimulation did not produce TNF α or IL-12 (Figure 3.5 A-B, Figure 3.10 A-B, Figure 3.16 A-B). These findings support a role for M1 macrophages in disease and the beneficial effect of generating more M2 macrophages for therapy.

By comparison, PAM3 stimulated human cells did produce elevated levels of the suppressive cytokine IL-10 (Figure 3.5 C, Figure 3.10 C, Figure 3.16C). The role of IL-10 in autoimmunity is controversial, with both beneficial and harmful effects being described in human and mice models of lupus. IL-10 treatment can result in the activation/proliferation of B cells [324] yet it impedes APC activation. In murine lupus, IL-10 treatment was found to alleviate disease (although its effect in human lupus remains controversial) [276, 325, 326]. IL-10 secretion observed in PAM3 treated macrophages is an indication of their M2-like phenotype but the implication for disease severity is unclear. IL-10 was significantly increased in the splenocyte cultures of PAM3 treated NZB/W mice. Since a significant drop in serum autoantibody levels was observed (consistent with a reduction in B cell activation), I

conclude that increased IL-10 levels did not cause harm. In both human and mice studies it was shown that PAM3 stimulation did not suppress B cell activation directly. Rather, it induced the differentiation of IgG producing cells [327]. Thus, it is possible that the increased endocytic activity of PAM3 generated macrophages coupled with no increase in CD40 expression resulted in IL-10 failing to stimulate B cells (Figure 3.18 A, D).

The availability of murine models of disease are important as they facilitate the development and testing of novel therapies. There are many strains of lupus-prone mice available that allow studies in both genetic and inducible models. One of the most widely known and accepted models of lupus is the NZB/W mouse. Female NZB/W mice develop a disease clinically resembling human lupus. While there are some murine models of myositis, none replicate the major clinical features of human disease. Rather, they only mimic a portion of the symptoms like muscle weakness and cell infiltration. Thus, I was restricted to the use of lupus mice to investigate the therapeutic utility of PAM3 *in vivo*.

In this lupus model, disease severity correlates with the level of serum cytokines that promote monocyte infiltration (e.g. MCP-1, CX3CL1) and macrophage differentiation/proliferation (e.g. CSF-1) [241, 242]. The importance of macrophages in the pathogenesis of disease was established by studying the effect of macrophages depletion by knocking out CSF-1, its receptor or by systemic administration of the selective CSF-1R inhibitor; GW2580. Treatment of MRL/lpr lupus prone mice with MCP-1 or CX3CL-1 antagonists reduced proteinuria and kidney damage but did alter autoantibody levels. Li et al reported that the adoptive transfer of M2 macrophages also halted disease progression, a finding that demonstrates the beneficial effects of M2 macrophages in lupus [251]. Unfortunately transfer of M2-like macrophages is not a practical clinical strategy. Rather than depleting all macrophages (which will have side effects such as increasing host susceptibility to infection), I examined whether increasing the frequency of M2 macrophage could be of use in the treatment of autoimmunity.

PAM3 was injected i.p. into NZB/W mice based on a schedule and dose previously reported to be effective in the treatment of allergic asthma and cardiac dysfunction in other animal models [260-262]. PAM3 treatment was started either at 7 wk of age

(before kidney damage developed) or at 13 wk of age at which time proteinuria was present. Macrophages were identified on the basis of 25F9 expression while M2 macrophages also expressed the CD206 mannose receptor. In the absence of any treatment, the ratio of M1:M2 macrophages rose as disease progressed in these lupus prone animals. Consistent with the in vitro findings obtained using human monocytes, in vivo treatment of NZB/W mice with PAM3 resulted in the preferential expansion of M2 macrophages and a normalization of the M1:M2 balance (Figure 3.17 A, B). Meanwhile, various measures of kidney function, including proteinuria and the BUN:creatinine ratio, showed that kidney damage was significantly reduced in both groups of PAM3 treated mice (Figure 3.19 A-D). Histological evaluation of kidneys by PAS staining confirmed that glomerular and tubular infiltration were reduced. This reduction in glomerulonephritis was accompanied by an increase in the frequency of M2 macrophages in the kidneys (Figure 3.20 A-G). These findings support our prediction that PAM3 can be used to generate M2 macrophage that reduce disease severity in NZB/W mice.

Survival was also prolonged by PAM3 treatment. By the age of 40 weeks, >60% of PAM3 treated NZB/W mice were alive whereas only 5 % were alive in the PBS control group (Figure 3.21). Late initiation of PAM3 therapy had a more modest effect on survival. Late treatment also failed to reverse proteinuria, indicating that PAM3 might slow the progression of lupus but could not reverse pre-existing kidney damage.

Studies of human monocytes showed that PAM3 induced them to mature into macrophages that were phenotypically and functionally M2-like. This was observed using with samples from healthy controls and from patients with various autoimmune/inflammatory diseases. Additionally, the macrophages generated by PAM3 showed increased expression of surface markers such as CD163, CD44 and CD40 whose function should reduce disease severity in autoimmune conditions. The increased endocytic activity of these M2-like macrophages reduced one of the drivers of continued autoantibody production. Inflammatory cytokines that can also contribute to disease severity decreased as a result of M2-like macrophage generation. In vivo studies performed in NZB/W mice supported the potential clinical utility of PAM3 to ameliorate disease. The observed reduction in disease

severity was established in studies of proteinuria, BUN:creatinine ratio, autoantibody and pro-inflammatory cytokine production. Of greatest importance, survival was prolonged and kidney damage was significantly delayed. An increased frequency of M1 macrophages in the kidney correlates with disease severity in murine models of lupus [277]. Our results indicate that increasing the frequency of M2 macrophages in the kidneys leads to a decrease in glomerular damage. There is currently no therapy of autoimmune disease that targets monocyte/macrophages, yet these cell types are important contributors to disease pathogenesis as emphasized in this thesis. Thus, studies done in this thesis indicate that using PAM3 to generate M2 macrophages has the potential of being a novel and effective therapeutic approach to autoimmune disease.

Extensive preclinical and clinical studies have shown that CpG ODN are effective immunostimulatory candidates to be used in a variety of applications such as vaccine development as adjuvants or in allergic and autoimmune disorders. Despite their potential as adjuvants, utility of free CpG ODN faces many disadvantages. They are prone to DNase digestion and protein adsorption which decreases their efficacy in in-vivo applications. Liposomes are promising candidates to be used as delivery systems that will improve the cellular uptake of its cargo and protect it from digestion. Encapsulation of these CpG ODN's within proper depot systems improve their bioavailability and bioactivity of the target cell. We have shown before that, liposomal encapsulation of CpG ODN's enhance their in vivo immunostimulatory activity [72]. It was also reported that cellular uptake was increased along with the antigen presentation [78]. It was shown that loading D-type CpG ODN into neutral or anionic liposome and K-type CpG ODN into cationic liposomes was more efficient in terms of encapsulation and enhanced immunomodulatory effects.

It is established in the literature that D-type CpG ODN induces secretion of IFN α from human pDCs by localizing to early endosome whereas K-type CpG ODN results in TNF α secretion through its rapid localization to late endosome [63, 279-283]. Previously it was also shown that, when D and K- type ODN are mixed and introduced to cells in their natural forms only K-specific response is observed [284]. Rapid localization of K-type CpG ODN to late endosome, resulting in TNF α

secretion suppresses the secretion of IFN α by D-type CpG ODN. In order to prove enhanced immunomodulatory effects of CpG ODNs upon liposomal encapsulation, I measured their ability to induce both CpG specific responses upon simultaneous stimulations. Combination of NL(D) and CL(K) resulted in significantly increased IFN α production from pDCs, which was lost upon stimulation with CpG ODNs natural forms (Figure 1). Thus, liposomal encapsulation overcame the blocking of the D-specific responses by K-type CpG ODN. Along with enhanced cytokine secretion, adjuvants should also have induced the maturation and activation of APC. Thus, co-stimulatory molecule analysis showed that, liposomal combination of D- and K-type CpG ODN induced enhanced APC functions, indicating that they have potent adjuvant effects overall (Figure 1C).

Secretion of type I interferons are important in the regulation of innate and adaptive immune responses, as they induce the maturation of DC, establishment of effector and memory T cell responses leading to the activation of humoral immunity [328]. Thus, type I IFN inducers are considered as potential vaccine adjuvants. D-type ODN tend to form complex multimers in solution due to interactions between their poly-G tails, and thus has posed a barrier to their use in clinical trials. However, encapsulation of D-type CpG ODN into stable virus like particles (VLP) and used as adjuvants in preclinical and clinical studies [329-331]. Additionally, researches have been investigating methods to combine both CpG specific effects by modifying K-type CpG ODN such as, combining K-type with SPG or forming nanoring with peptides for the induction of type I IFNs [332, 333]. We hypothesized by encapsulating D-type CpG ODN into liposomes, it will eliminate the problems caused by its varying size and thus leading to a more stabilized and consistent immune activation. It was interesting that liposomal encapsulation also eradicated the blocking problem that was observed by the simultaneous stimulation of D and K-type CpG ODNs in their natural forms by inducing both IFN α and TNF α secretion. Thus, I speculated that this could be due to either altered sub-cellular localization of either CpG ODN or their uptake kinetics. In order to understand the underlying mechanism of liposomal encapsulation resulting in both specific effects, I investigated the subcellular localization of both alone forms of liposomal CpGs, and their combinations. It was clear that there was no difference in their localization both in alone or combinations forms of liposomes. On the other hand, cellular

internalization studies revealed that encapsulation of K-type CpG ODN delayed its internalization, most likely due to the increased size, whereas internalization of D-type CpG in neutral liposome increased its internalization by human pDCs.

Human and mice differ in the cells that express TLR9 which leads to changes in their response to CpG ODNs. In humans, only pDC and B cells express TLR9 whereas in mice DCs and macrophages also express it [285]. So, before going into *in vivo* experiments, I investigated the effects of liposomal combinations on mice splenocytes. Mice results correlated with human results, indicating that the liposomal combination was effective in both.

After establishing the altered immunomodulatory effects of NL(D)+CL(K) to try its potency as a vaccine adjuvant I started an *in vivo* trial. Liposomal CpG ODNs also included OVA antigen and BL6 mice were immunized with the formulations twice in two weeks apart. OVA specific responses were measured from the sera of mice in order to establish the type of adaptive response. Mice produced 5 folds higher IgG2c, resulting in a Th1-biased humoral immunity ($\text{IgG2c/IgG1} > 1$). Next mice were challenged with OVA expressing melanoma cell line B16. And correlating with increased anti OVA specific responses, tumor formation was greatly delayed in the groups that received liposomal CpG injection.

Liposomal encapsulation of both CpG ODNs, made it possible to combine the effects of both CpG types along with an improvement in both $\text{IFN}\alpha$ and $\text{TNF}\alpha$ production as well as co-stimulatory molecule upregulation. Combination of liposomes induced an enhanced Th1 biased OVA specific humoral response that helped to control the tumor development. The combination we have developed demonstrates that the beneficial features of both CpG ODNs can be obtained by appropriate liposomal formulation, which further extends the breadth of CpG ODN dependent immunotherapy.

Chapter 5

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Chapter 6

Curriculum Vitae and Publications

PERSONAL INFORMATION

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EDUCATION

2017-2019 Visiting Fellow, Cancer and Inflammation Program, National Cancer Institute, National Institutes of Health, Frederick, MD

2014- 2019 *Ph.D. Candidate*, Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

2014 *MSc.*, Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

2012 *B.S.*, Chemistry, Istanbul Technical University, Istanbul, Turkey

2009-2010 *B.S.*, Chemistry, Erasmus Exchange Program, Universidad de Valencia, Valencia, Spain

RESEARCH EXPERIENCE

2017-2019 Visiting Fellow, Cancer and Inflammation Program, National Cancer Institute, National Institutes of Health, Frederick, MD
Principal Investigator: Dr. Dennis M. Klinman

2014-2019 PhD Candidate, Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

Principal Investigator: Prof. Dr. Ihsan Gursel
PhD Co-Advisor: Dennis M. Klinman
Project:

- 2012-2014 MSc Student, Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey
Principal Investigator: Prof. Dr. Ihsan Gursel
- 2009-2012 Laboratory of Biopolymers, Department of Organic Chemistry, Istanbul Technical University, Istanbul, Turkey
Principal Investigators: Prof. Dr. Umit Tunca & Prof. Dr. Gurkan Hizal
- 06-08/2012 Summer Intern, Laboratory of Polymers, Department of Chemistry, TUBITAK MAM Research Center

TEACHING EXPERIENCE

- 2018 Journal Club for Summer Students, NCI, NIH, Frederick, USA
- 2012-2017 Graduate Teaching Assistant, Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey
- 2008-2009 Student Teaching Assistant, Department of Chemistry, Istanbul Technical University, Istanbul, Turkey

AWARDS AND SCHOLARSHIPS

- 2019 AAI Trainee Poster Award, Immunology 2019, San Diego, USA 9-13 May 2019
- 2016 FIMSA Travel Award for the 45th Annual Meeting of JSI (Japanese Society of Immunology), 5-7 December 2016, Okinawa, Japan.
- 2016 Partial Fellowship by EFIS-EJI (European Federation of Immunological Society and European Journal of Immunology) for EFIS-EJI Ruggero Ceppellini Advanced School of Immunology (12-14 October 2016) Naples, Italy.
- 2016 Turkish Society of Immunology Bursarie for 3rd Molecular Immunology & Immunogenetics Congress, Antalya, Turkey, April 2016
- 2015 Turkish Society of Immunology Bursarie for 4th European Congress of Immunology, Vienna, Austria, September 2015

- 2014-2016 Project bursary, Ministry of Industry and Trade (SANTEZ *0566-STZ-2013-2* grant), “Enhancement of FMD vaccine formulations immunostimulatory effects using CpG ODNs” June 2014-June 2016 (Duration: 24 months)
- 2014 Turkish Society of Immunology Bursarie for 2nd Molecular Immunology & Immunogenetics Congress, Antalya, Turkey, April 2014

PROFESSIONAL MEMBERSHIPS

- 2012-Present Turkish Society of Immunology
2018-Present The American Association of Immunologists

PRESENTATIONS

Oral Presentations

Begum H. Horuluoglu, Dennis Klinman. PAM3 supports the generation of M2-like macrophages from lupus patient monocytes and improves disease outcome in murine lupus Cancer and Inflammation Program, NCI, NIH, USA December 2018

Begum H. Horuluoglu, Dennis Klinman. Effect of PAM3 on monocyte differentiation in SLE. Cancer and Inflammation Program, NCI, NIH, USA February 2018

Begum H. Horuluoglu, Ihsan Gursel. Liposomal D-and K-type CpG ODN combination confers synergistic immune activation and act as an efficient tumor vaccine. 3rd Molecular Immunology & Immunogenetics Congress, Antalya, Turkey, April 2016

Poster Presentations

Begum H. Horuluoglu, Defne Bayik, Neslihan Kayraklioglu, Emilie Goguet, Mariana Kaplan, Dennis M. Klinman_PAM3 supports the generation of M2-like macrophages from lupus patient monocytes and improves disease outcome in murine lupus. Immunology 2019, San Diego, CA, USA, May 2019.

Begum H. Horuluoglu, Defne Bayik, Emilie Goguet, Debra Tross, Luz P. Blanco, Mariana Kaplan, Dennis M. Klinman. Modulation of TLR2/1 by PAM3CSK4 to induce generation of immunosuppressive macrophages as a therapeutic approach for SLE. Immunology 2018, Austin, TX, USA, May 2018.

Begum H. Horuluoglu, Defne Bayik, Emilie Goguet, Debra Tross, Luz P. Blanco, Mariana Kaplan, Dennis M. Klinman. Effects of toll Like receptor-2/1 agonist PAM3CSK4 on monocyte differentiation of SLE patients and investigating its role as a therapeutic agent. 14th Annual NIH Graduate

Student Research Symposium, National Institutes of Health, Washington DC, USA, February 2018.

Begum H. Horuluoglu, Ihsan Gursel, Dennis M. Klinman. Overriding K-and D- type CpG ODN dichotomy: Encapsulating K-type and D-type CpG ODNs within liposome induce enhanced immune activation and act as an efficient tumor vaccine formulation. Cancer Immunology and Immunotherapy: From Conception to Delivery, National Institutes of Health, Washington DC, USA, October 2017.

Begum H. Horuluoglu, Ihsan Gursel. Dennis M. Klinman Overriding K-and D- type CpG ODN dichotomy: Encapsulating K-type and D-type CpG ODNs within liposome induce enhanced immune activation and act as an efficient tumor vaccine formulation. 2017 Immunology Interest Group Training Workshop, National Cancer Institute, Leesburg VA, USA, September 2017.

Begum H. Horuluoglu, Ihsan Gursel. Overriding K-and D- type CpG ODN dichotomy: Encapsulating K-type and D-type CpG ODNs within liposome induce synergistic immune activation and act as an efficient tumor vaccine formulation. 45th Annual Meeting of JSI, Okinawa, Japan, December 2016

Begum H. Horuluoglu, Ihsan Gursel. Liposome encapsulation overcomes D-type and K-type CpG ODN dichotomy and induces synergistic immune activation. ILS Liposome Advances 2015, London, United Kingdom December 2015

Begum H. Horuluoglu, Ihsan Gursel. Liposome encapsulation overcomes D-type and K-type CpG ODN dichotomy and induces synergistic immune activation. 4th European Congress of Immunology, Vienna, Austria September 2015

Begum H. Horuluoglu, Ihsan Gursel. Synergistic Immune Activation of liposome encapsulated D- and K- type ODNs. 23th National Congress of Immunology, Antalya, Turkey, April 2015

Begum H. Horuluoglu, Ihsan Dereli, Banu Bayyurt, Gozde Gucluler, Kubra Almacioglu, Ihsan Gursel. Delineation of Synergistic Immunostimulatory Effects of Liposomal D- and K-Type CpG ODNs on Human PBMC, 2nd Molecular Immunology & Immunogenetics Congress, Antalya, Turkey, April 2014

Gozde Gucluler, Tamer Kahraman, Defne Bayik, Begum H. Horuluoglu, Arda Gursel, Ihsan Gursel. CpG ODN Loaded Extracellular Nanovesicles: Enhanced Immunotherapeutic Activity, ILS Liposome Advanced Conference, London, United Kingdom, 13-17 December 2013

B.Bayyurt, G. Gucluler, D. Bayik, E. Alpdundar, S.Yildiz, B.H. Horuluoglu, M.Ozcan, M. Gursel, I. Gursel. Extended anti-cancer immunity by liposomes coencapsulating nucleic acid TLR-ligands and antigen. 15th International Congress of Immunology, Milan, Italy, August 2013

PUBLICATIONS

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PAM3 supports the generation of M2-like macrophages from lupus patient monocytes and improves disease outcome in murine lupus

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ABSTRACT

Systemic Lupus Erythematosus (SLE) is an autoimmune syndrome of unclear etiology. While T and B cell abnormalities contribute to disease pathogenesis, recent work suggests that inflammatory M1-like macrophages also play a role. Previous work showed that the TLR2/1 agonist PAM3CSK4 (PAM3) could stimulate normal human monocytes to preferentially differentiate into immunosuppressive M2-like rather than inflammatory M1-like macrophages. This raised the possibility of PAM3 being used to normalize the M1:M2 ratio in SLE. Consistent with that possibility, monocytes from lupus patients differentiated into M2-like macrophages when treated with PAM3 *in vitro*. Furthermore, lupus-prone NZB x NZW F1 mice responded similarly to weekly PAM3 treatment. Normalization of the M2 macrophage frequency was associated with delayed disease progression, decreased autoantibody and inflammatory cytokine synthesis, reduced proteinuria and prolonged survival in NZB x NZW F1 mice. The ability of PAM3 to bias monocyte differentiation in favor of immunosuppressive macrophages may represent a novel approach to the therapy of SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune syndrome characterized by periods of intense inflammation affecting multiple organ systems (most notably the kidneys, skin and CNS) in association with the production of autoantibodies targeting nucleic acids and proteins that bind to nucleic acids [1,2]. Lupus is a multifactorial disease whose onset and severity are influenced by both genetic and environmental factors. Current treatments include antimalarials, corticosteroids and various immunosuppressive drugs [3]. These are largely but not universally effective at controlling disease flares and their prolonged use has undesirable side effects. The development and testing of novel therapies for lupus is facilitated by the availability of spontaneous murine models of the disease. In particular, NZB x NZW F1 mice (hereafter NZB/W) develop lupus-like disease whose symptoms and gender bias are similar to those seen in humans [4,5]. The immune abnormalities manifested by female NZB/W mice also mimic those of lupus patients including autoantibody production, lymphocyte activation and changes in monocyte/macrophage frequency [6].

Monocytes constitute 5–10% of peripheral blood leukocytes and

function to protect the host from infection by differentiating into macrophages and dendritic cells [7]. Monocytes are typically classified into 3 subtypes based on their phenotype and function. Those that express CD14 but not CD16 are termed ‘classical’ monocytes. These are phagocytic but do not support inflammatory responses. ‘Non-classical’ monocytes express a CD14^{low}CD16⁺ phenotype and are inflammatory. An ‘intermediate’ or transitional monocyte population also exists that is CD14⁺CD16⁺ and has both phagocytic and inflammatory properties [7]. In response to stimulation, monocytes differentiate into macrophages with diverse phenotypes and functions. These range from ‘classical’ pro-inflammatory M1-like macrophages that protect the host from infection to ‘alternatively activated’ M2-like macrophages that are immunosuppressive, support tissue remodeling, and phagocytose apoptotic cells [8–10].

Recent studies suggest that monocytes and macrophages can contribute to the pathogenesis of SLE. Indeed, lupus flares are associated with a relative increase in the frequency of M1 vs M2 macrophages [11]. The frequency of inflammatory monocytes is also elevated in SLE patients and the concentration of serum cytokines that promote macrophage differentiation (such as CSF-1 and MCP-1) correlates with disease severity [12]. Normalizing the M1:M2 ratio by adoptive transfer of M2 macrophages is of benefit

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in the treatment of murine lupus [13]. Hence, efforts to increase the frequency of immunosuppressive M2-like macrophages represents a potentially useful strategy for the treatment of SLE.

Our lab previously showed that the TLR2/1 agonist PAM3 was unique among TLR ligands in promoting the generation of M2-like macrophages from normal human monocytes [14,15]. This work extends those findings to show that PAM3 similarly induces monocytes from lupus patients to preferentially differentiate into M2-like macrophages. Weekly *in vivo* treatment of female NZB/W mice with PAM3 normalized the M1:M2 ratio in those animals. Treatment was accompanied by immunologic changes including a reduction in IgG anti-DNA autoAb and inflammatory cytokine levels and clinically by decreased proteinuria and prolonged survival. The ability of PAM3 to preferentially support the generation of immunosuppressive M2 rather than inflammatory M1 macrophages represents a novel approach to the therapy of SLE.

2. Materials & methods

2.1. Isolation and differentiation of human monocytes

Blood was obtained from SLE patients and healthy volunteers after obtaining IRB-approved written consent (NIDDK/NIAMS IRB, Bethesda, MD). PBMC were isolated by density centrifugation over Histopaque-1077 (Millipore Sigma, Merck) at 400 g for 30 min. Monocytes were sorted using human CD14 Microbeads (MACS, Miltenyi Biotec) according to the manufacturers' protocol. Briefly, 20 μ l of CD14 microbeads were added to 10^7 cells suspended in 80 μ l of MACS buffer. After 15 min incubation in the dark at 4 °C cells were washed, re-suspended in 500 μ l of MACS buffer and loaded onto magnetized LS columns. The columns were extensively washed and CD14⁺ cells collected by removing the magnet and flushing with 5 ml of MACS buffer.

10^5 purified monocytes/well were seeded into 96-well flat bottom plates (Corning) and stimulated for 5 days at 37 °C with 1 μ g/ml PAM3CSK4 (PAM3) (Invivogen), 250 ng/ml MCSF (Miltenyi Biotec), 250 ng/ml IFN γ (Miltenyi Biotec) or 1 μ l/ml R848 (Invivogen). Previous studies established that these were the optimally stimulatory concentrations of each agent ([14,15] and data not shown). Cells were maintained in medium consisting of RPMI 1640 (Lonza) supplemented with 2% heat-inactivated fetal calf serum 25 nmol/L HEPES, 1 mmol/L sodium pyruvate, non-essential amino acids (NEAAs) and 0.0035% 2-mercaptoethanol (2-ME).

2.2. In vivo experiments

Female Balb/C, Female NZB and male NZW mice purchased from The Jackson Laboratory (Bar Harbour, ME) were bred. Female NZB/W F1 offspring were housed under pathogen-free conditions in the NCI animal facility. All experiments were reviewed and approved by the NCI Animal Care and Use Committee. Lupus-prone NZB/W mice were injected weekly with 100 μ g of PAM3 (Invivogen) i.p. starting early (7 wk of age) or late (13 wk).

The protein content of early morning urine was monitored using Albustix test strips (Siemens Heathineers). BUN and creatinine levels were measured in serum of 6 month old mice by ELISA (Bioassay systems and ThermoFischer, respectively) according to the manufacturers' protocol.

Two long term studies were performed in NZB/W mice. In the first, 5 mice/group were treated with PAM3 starting at either 7 or 13 weeks of age. Surviving mice were sacrificed at 6 months of age to assess the phenotype of macrophages and cytokine production. In the second experiment, 6 mice/group were treated as described above and survival monitored through 45 weeks. An excess of controls (NZB/W mice treated with PBS group) were included in these experiments. Survival was monitored until 45 weeks of age. Mice were sacrificed only when deemed in extremis by veterinary staff.

Single cell suspensions were prepared from peritoneal and spleen cells of sacrificed mice and cultured in medium consisting of RPMI 1640 (Lonza) supplemented with 5% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin 25 nmol/L HEPES, 1 mmol/L sodium pyruvate, NEAAs and 0.0035% 2-ME. Mice were bled to obtain sera for the evaluation of cytokine and auto-antibody levels.

2.3. Kidney histology

Kidney damage was evaluated in 2–3 μ m formalin fixed paraffin embedded sections using Periodic Acid Schiff (PAS) and Masson trichrome staining. Pathological changes evaluated included glomerular activity (i.e., glomerular proliferation, karyorrhexis/fibrinoid necrosis, cellular crescents and hyaline deposits), tubulointerstitial activity (i.e., tubular dilatation and interstitial inflammation) and chronicity of the lesions (i.e., glomerulosclerosis, tubular atrophy, interstitial fibrosis) as previously reported [16]. Glomerular activity was evaluated on 30 glomeruli/sample and scored using 0–3 scale in which 0 = no lesions, 1 = lesions in < 25% of glomeruli, 2 = lesions in 25–50% of glomeruli and 3 = lesions in > 50% of glomeruli. Tubular activity and chronicity indices were based on a 0–4 scale as 0 = no lesions, 1 = lesions in 1–10%, 2 = 11–25%, 3 = > 25–50% and 4 = > 50%. Glomerulonephritis was calculated using the mean scores for each pathological feature. The infiltration of M2 macrophages into these kidneys was determined by immunohistochemical staining of formalin fixed sections stained with antibodies specific for F4/80 and CD206.

2.4. Flow cytometry

Human cells were stained with fluorochrome-conjugated Abs specific for human CD16, 25F9 (eBioscience), CD163, CD40 and/or CD14 (Biolegend) for 20 min in PBS – 2% BSA on ice. Murine cells were first incubated with Fc Block (Biolegend) for 15 min and then stained with fluorochrome conjugated Abs specific for murine CD45, CD11b, F480 and CD206 under the same conditions. Stained cells were washed and fixed with Fix & Perm Medium A (Invitrogen).

To detect intracellular IL-12, cells were cultured overnight with a 1:1000 dilution of GolgiPlug (BD Biosciences), stained in Fix & Perm Medium B for 20 min with fluorochrome-conjugated anti-IL-12 (BDBiosciences). All stained cells were washed, re-suspended in PBS – 2% BSA and analyzed using an LSR Fortessa (BD Biosciences).

2.5. Endocytosis assay

Human macrophages that were stimulated for 5 days, were then incubated with 25 μ g/ml of pH-Rodo Zymosan (Invitrogen) particles or medium (as background control) for 45 min at 37 °C. Cells were then washed and stained with 25F9 and CD163. Uptake was calculated by subtracting background from pH-Rodo Zymosan levels determined by LSR Fortessa.

2.6. ELISA

Immunol 2HB-microtiter plates were coated with human anti-TNF α (R&D Systems), mouse anti-IL12 (BD Biosciences), mouse anti-TNF α (R&D Systems) or mouse anti-IL-10 (R&D Systems) and then blocked with 2% BSA-PBS for 2 h. After washing, culture supernatants or serially diluted sera were added overnight at 4 °C. Plates were washed, incubated with biotin-labeled secondary Ab and developed by adding phosphatase-conjugated streptavidin (AKP) followed by p-nitrophenyl phosphatase (pNPP) substrate (Southern Biotech). Optical density was measured using a SpectraMax M5 microplate Reader and SoftMax Pro Acquisition and Analysis software (Molecular Devices).

IgG anti-dsDNA was detected by coating Immunol 2HB-microtiter plates with 4 μ g/ml of CT-DNA (Sigma) in DNA binding solution (Abcam) for 4 h. The plates were blocked with 2% BSA-PBS and

Table 1
Characteristics of SLE patients.

Number of patients	35
Age	46 ± 13
Gender	(N and %)
Female	32 (91%)
Male	3 (9%)
SLEDAI-2K	
< 6	30 (85%)
> 6	5 (15%)
Medications	
HCQ	27 (77%)
Prednisone	26 (74%)
AZA	7 (20%)
MMF	9 (26%)

The SLEDAI-2K scoring system was used to evaluate disease severity. Those with scores > 6 were considered to have active disease [60,61]. Medications included HCQ (hydroxychloroquine), AZA (azathioprine), and MMF (mycophenolate mofetil).

incubated with diluted mouse serum as above. Bound Ab was detected with biotin labeled IgG (Invitrogen) followed by phosphate-conjugated streptavidin and pNPP substrate.

2.7. Statistical analysis

Statistical analysis was done using 2-tailed unpaired Student t-tests or Dunnett corrected One-way ANOVA multiple comparison tests (GraphPad Software Inc.).

3. Results

3.1. Frequency of inflammatory monocytes in lupus patients

Human monocytes are conventionally classified into 3 subsets based on their activity and phenotype (see Introduction). The literature indicates that an imbalance in the relative frequency of monocyte subpopulations supports the development and persistence of autoimmune disease [6]. To clarify the role of monocytes in patients with SLE, PBMC from patients with both active and inactive disease (Table 1) were examined. Consistent with previous reports, the absolute number of monocytes in the peripheral blood of patients did not differ from normal controls [6] although the frequency of inflammatory monocytes (intermediate and non-classical) was significantly elevated in SLE patients (Fig. 1A and B). The frequency of inflammatory versus classical monocytes did not differ between patients with active versus inactive lupus (Supplemental Fig. 1).

3.2. PAM3 induces lupus monocytes to preferentially mature into phenotypically M2-like macrophages

Human monocytes can be stimulated to differentiate into macrophages that vary in both phenotype and function depending upon the nature of the stimulus [8,17]. While the best way to categorize these different types of macrophages remains controversial, for simplicity the scheme originally developed to describe murine macrophages will be used in this work [18]. Macrophages that are pro-inflammatory, promote Th1 responses and/or support anti-tumor immunity will be referred to as classically activated “M1-like” whereas those that down-modulate over-exuberant inflammatory responses, clear apoptotic cells and/or support tissue remodeling will be referred to as alternatively activated “M2-like” [19].

Previous studies showed that normal human monocytes preferentially matured into M1-like macrophages when stimulated by most

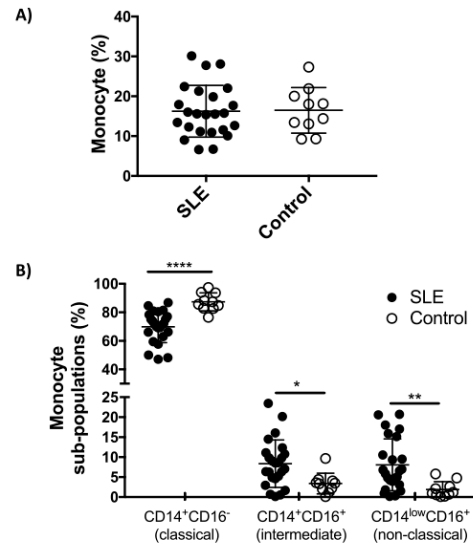


Fig. 1. Characterization of monocytes from patients with SLE. Data show A) the percent of monocytes in PBMC and B) the percent of each monocyte subpopulation in samples from 24 lupus patients and 10 healthy controls. **p* < .05; ***p* < .01; ****p* < .001.

TLR agonists [8,20]. The one exception to this rule was PAM3 which uniquely triggered human monocytes to mature into immunosuppressive M2-like macrophages [14,15]. Given the excess of inflammatory monocytes detected in the blood of lupus patients coupled with evidence from lupus-prone mice that boosting the frequency of M2 macrophages can reduce disease severity [13,21], experiments were undertaken to determine whether PAM3 could stimulate monocytes from lupus patients to mature into M2-like macrophages.

Preliminary experiments identified the optimal conditions for triggering the differentiation of freshly isolated human monocytes ([14,15] and data not shown). The maturation process was characterized by acquisition of 25F9, a surface marker uniquely expressed by mature human macrophage [22]. Consistent with earlier studies of normal human donors, monocytes from lupus patients preferentially differentiated into 25F9⁺ macrophages when cultured with PAM3 (*p* < .001, Fig. 2A, B). Other agents that support the generation of M2-like (MCSF) or M1-like (IFN γ or R848) macrophages were included as controls [14]. The majority of macrophages generated by PAM3 and MCSF expressed an M2-like phenotype characterized by the up-regulation of CD163 (a scavenger receptor preferentially expressed by M2-like macrophage) as well as 25F9 (*p* < 0.01, Fig. 2A–D). In contrast, the macrophages generated by R848 and IFN γ predominantly expressed the M1-like CD163⁻ 25F9⁺ phenotype (*p* < 0.05, Fig. 2A–D). There was no difference in the response of monocytes isolated from lupus patients vs normal controls in their response to PAM3, MCSF, R848 or IFN γ . As previously reported, M1-like macrophages also selectively up-regulated the co-stimulatory molecule CD40 (*p* < .001, Fig. 2E) [23]. It is noteworthy that although unstimulated monocytes generally die during *in vitro* culture, those from lupus patients survived 3-fold better and these preferentially differentiated into M1-like macrophages (*p* = .031, Fig. 2C and data not shown).

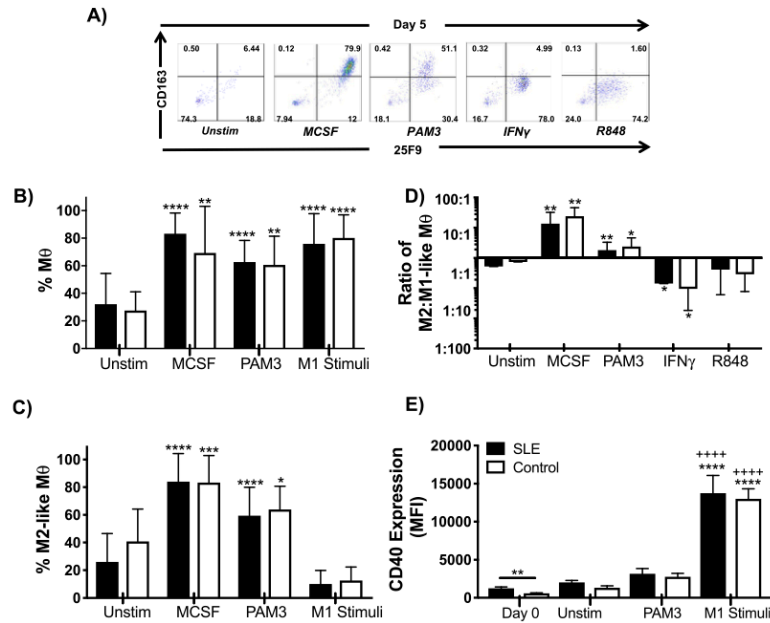


Fig. 2. Effect of PAM3 on the phenotype of lupus monocytes. Monocytes were MACS purified from the peripheral blood of lupus patients and normal controls and stimulated *in vitro* for 5 days with optimal concentrations of PAM3, M-CSF, IFN γ or R848. **A)** Representative histogram showing the expression of 25F9 and CD163 by cultured cells. Note that < 1% of purified monocytes expressed either marker before being placed in culture (day 0). **B)** Percent of cultured cells that acquired 25F9 expression, **C)** percent of 25F9 $^{+}$ cells that co-expressed the CD163 M2-like macrophage marker and **D)** ratio of CD163 $^{+}$ 25F9 $^{+}$: CD163 $^{-}$ 25F9 $^{+}$ cells. **E)** Mean fluorescence intensity of CD40 expression. Data derive from independent studies of 23 lupus samples (solid bars) and 9 normal controls (open bars) where R848 or IFN γ were used as the M1 stimuli. **p < .01; ***p < .001; ****p < .0001 compared to unstimulated cells from the same donors, whereas + + + + p < .0001 compared to Day 0.

3.3. PAM3 treatment induces lupus monocytes to preferentially mature into functionally M2-like macrophages

An important feature of M2-like macrophages is their ability to endocytose apoptotic debris [10]. Indeed, defective dead cell clearance reportedly contributes to the inflammation and autoAb production characteristic of lupus patients [24–27]. The ability of PAM3 to generate macrophage with endocytic activity was therefore evaluated. Results show that lupus monocytes treated with PAM3 acquired endocytic activity (reflected by their ability to internalize zymosan particles) whereas those treated with M1 stimuli did not (Fig. 3A). This mirrored the effect of PAM3 on monocytes from normal volunteers. In both lupus patients and controls, this gain in function correlated with increased expression of CD163 by PAM3 treated monocytes ($R^2 = 0.92$, Fig. 3B).

The production of pro-inflammatory cytokines such as TNF α and IL-12 is characteristic of inflammatory M1-like macrophages [8,28]. The production of these cytokines reportedly worsens disease in lupus patients [29]. The effect of PAM3 on cytokine production was monitored. As expected, macrophage generated by treatment with M1 stimuli secreted large amounts of TNF α and gained the ability to produce IL-12. Those cultured with PAM3 showed no increase in the production of those cytokines when compared to unstimulated controls (Fig. 3C, D). However, they did acquire the ability to produce the immunosuppressive cytokine IL-10 (Fig. 3E). In toto, these findings

support the possibility that PAM3 might be useful for generating M2-like macrophages for the treatment of SLE. As a precursor to human clinical evaluation, studies were initiated to examine the effect of PAM3 in a relevant murine model.

3.4. The imbalance of M1:M2 macrophages in lupus-prone NZB/W mice is normalized by treatment with PAM3

Initial experiments examined whether administering PAM3 to female NZB/W mice (a widely accepted model of human lupus) elicited the same alteration in macrophage phenotype *in vivo* as was observed using human cells *in vitro*. Towards that end, mice were injected weekly with 100 μ g of PAM3 i.p., a schedule and dose previously reported to be effective in the treatment of allergic asthma and cardiac dysfunction [30–32]. Therapy was initiated at either 7 or 13 wk of age, timepoints that were either before or after the onset of detectable kidney damage (increased proteinuria) [33].

To monitor the effect of treatment on the M1:M2 ratio, macrophages were obtained from the peritoneal cavity of 6 month old mice. Murine macrophages were identified by their expression of the F480 surface marker and M2 macrophages by the co-expression of the CD206 mannose receptor [22,34]. The ratio of M1:M2 macrophage was significantly elevated in untreated NZB/W mice when compared to control BALB/c mice (Fig. 4). This is consistent with previous reports showing that disease in NZB/W mice is worsened by an excess of inflammatory

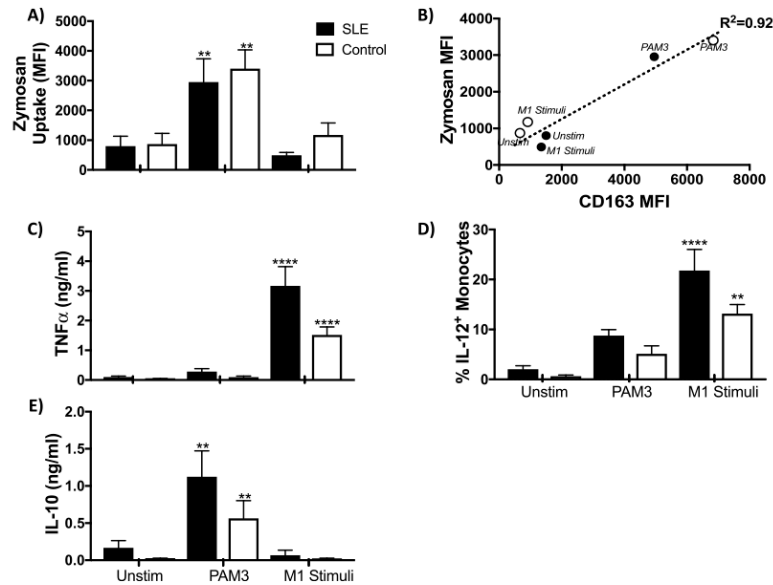


Fig. 3. Effect of PAM3 on the function of lupus monocytes. MACS purified monocytes were cultured as described in Fig. 2. A) The uptake of zymosan-labeled particles after 5 days of culture (N = 17 lupus samples and 6 controls). B) Correlation between the expression of CD163 and uptake of zymosan particles. C) Concentration of TNF α in culture supernatants. D) Percent of cultured cells staining for intracytoplasmic IL-12. E) Concentration of IL-10 in culture supernatants (N = 12 lupus samples and controls). **p < .01; ***p < .001; ****p < .0001 compared unstimulated cells from the same donors.

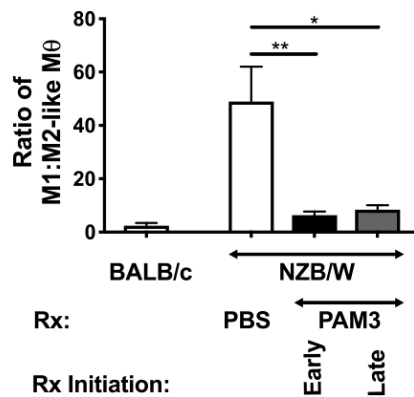


Fig. 4. Effect of PAM3 treatment on the M1:M2 ratio of NZB/W mice. Female NZB/W mice were treated weekly with 100 μ g of PAM3 starting either early (7 wk) or late (13 wk). Controls included littermates treated with PBS and normal (BALB/c) mice. The M1:M2 ratio was calculated based on the ratio of CD206 $^{+}$:CD206 $^{+}$ F480 $^{+}$ macrophages collected from the peritoneal cavity at 6 months of age. N = 5 NZB/W mice/group, N = 2 BALB/c controls. *p < .05; **p < .01; ***p < .001 for PAM3 vs PBS treated NZB/W mice.

macrophages [35]. The M1:M2 ratio was normalized in adult mice following PAM3 treatment starting at either 7 or 13 weeks of age (p < 0.01 for the early and < 0.05 for the late treatment group).

3.5. Effect of PAM3 treatment on the immune milieu of NZB/W mice

Additional immune manifestations of lupus in NZB/W mice included the production of autoAbs and inflammatory cytokines [5]. Consistent with the hypothesis that PAM3 might be useful in the treatment of SLE, serum IgG anti-DNA autoAb levels were 3–4 fold lower in the PAM3 vs PBS treated groups (p < 0.04 and < 0.01 in the early and late treatment groups respectively, Fig. 5A). This was accompanied by a significant decline in serum IL-12 levels and the amount of TNF α produced by spleen cell cultures (p < .001, Fig. 5B, C). Conversely, splenocytes from mice treated from 7 wk of age with PAM3 produced significantly more IL-10 (an immunosuppressive cytokine derived from M2 macrophages) than did untreated controls (p < .01, Fig. 5D). Spleen cells from mice whose treatment was delayed until wk 13 also produced more IL-10 but this effect did not reach statistical significance.

3.6. Clinical effect of PAM3 treatment

The effect of PAM3 treatment initiated either before or after the onset of proteinuria was monitored in two independent experiments. PAM3 reduced proteinuria in both experiments and both groups (p < .005 in the early and < 0.01 in the late treatment groups,

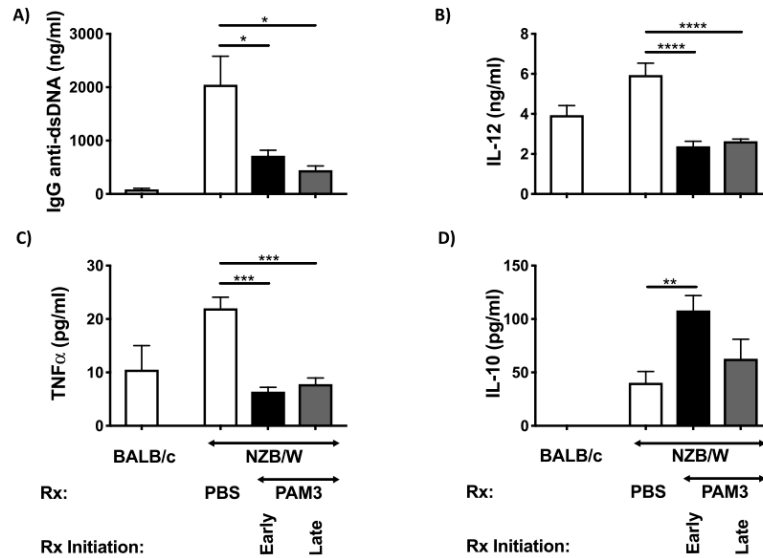


Fig. 5. Effect of PAM3 treatment on the immune manifestations of lupus. Mice were treated as described in Fig. 4. Serum collected at 6 months was analyzed for (A) IgG anti-dsDNA and (B) IL-12. Spleen cells were cultured ex vivo without further stimulation for 24 h and culture supernatants tested for (C) TNF α and (D) IL-10 levels by ELISA (N = 5/group of lupus mice and 2 for BALB/c controls). **p < .01; ***p < .001; ****p < .0001 PAM3 vs PBS treated NZB/W mice.

Fig. 6A). It also normalized the BUN:creatinine ratio that reached abnormally high levels as glomerulonephritis developed in control NZB/W mice (Fig. 6B).

The natural history of disease was examined by monitoring kidney histology and animal survival. Immunohistochemical staining of kidneys from control NZB/W mice showed that number of CD206⁺ M2 macrophages remained low as disease progressed (Fig. 7A and B). By comparison, the number of M2 macrophages in the kidneys of PAM3 treated mice increased over time consistent with the normalization of M1:M2 ratio observed in Fig. 4. Histological analysis of these kidneys confirmed that glomerular and tubular infiltration by inflammatory cells was delayed by PAM3 treatment (Fig. 7C–G). Of greatest import, PAM3 treatment significantly prolonged survival, with > 60% of mice whose treatment starting at 7 wk of age surviving through 11 months of age vs 5% in the control group (p = .0037, Fig. 8). Late initiation of therapy was less effective at slowing disease progression.

4. Discussion

This work establishes that the TLR 2/1 agonist PAM3 induces monocytes from lupus patients to preferentially differentiate into M2-like macrophages capable of suppressing autoimmune disease. The therapeutic potential of PAM3 was examined in the NZB/W model of lupus. Weekly treatment with PAM3 normalized the M2:M1 ratio and shifted the cytokine profile towards the production of suppressive rather than inflammatory factors. Serum levels of anti-dsDNA auto-antibodies fell, glomerulonephritis was delayed, and survival was prolonged by PAM3 therapy.

The conventional treatment of SLE relies on a combination of immunosuppressive drugs, steroids, and agents that deplete/inhibit autoreactive B cells [3,36,37]. Novel treatments designed to inhibit interferon or lymphocyte signaling are being evaluated in phase III clinical trials [37]. The over-abundance of pro-inflammatory M1-like

macrophages present in SLE patients provides an additional target for therapeutic intervention [11,12,38–40]. In the MRL/lpr model, depleting macrophages by knocking out CSF-1, its receptor, or by systemic administration the selective CSF-1R inhibitor GW2580 reduced disease severity [12,41–44]. Proteinuria and glomerular damage (but not autoAb levels) were also reduced when monocyte infiltration was inhibited by treating MRL/lpr or NZB/W mice with agents that antagonized macrophage differentiation/proliferation [44,45].

The dominance of M1-like macrophages in lupus patients is likely driven by the increased level of pro-inflammatory cytokines including IL-12, TNF α , IFN γ and type I IFNs present in that disease. These support the generation of M1-like macrophages via pathways involving JAK/Stat-1, NFkB and interferon regulatory factors [46–48]. Chemokines that promote monocyte infiltration (e.g. MCP-1, CX3CL1) and macrophage differentiation/proliferation are also elevated in mice and patients with lupus and may contribute to this process [12,41].

While murine studies show that reducing overall macrophage abundance slows disease progression, Li et al. showed that increasing the frequency of M2 macrophages accomplished the same goal [13]. That group adoptively transferred M2 macrophages into lupus prone mice and documented a significant reduction in disease severity [13]. Unfortunately, adoptive M2 macrophage transfer is not a practical clinical strategy. We therefore examined whether treatment with PAM3, a TLR agonist that induces normal human monocytes to differentiate into M2-like macrophages [14], might be used to normalize the M1:M2 ratio in SLE patients. As shown in Fig. 2C, that outcome was obtained in studies of cultured lupus monocytes. In the absence of exogenous stimulation, lupus monocytes preferentially mature into M1-like macrophages (Fig. 2D). This may reflect their *in vivo* exposure to factors that support the generation of inflammatory macrophages, as described above. Just the opposite is observed in cancer patients, whose monocytes respond to suppressive serum factors by preferentially maturing into M2-like macrophages [49,50].

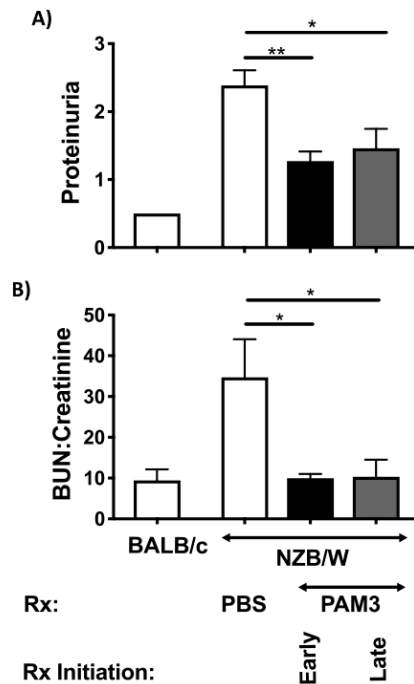


Fig. 6. Effect of PAM3 treatment on lupus nephritis. NZB/W mice were treated with PAM3 or PBS as described in Fig. 4. A) Proteinuria was measured at 6 months (N = 11 for each PAM3 treated group and 21 for PBS treated mice). B) The serum BUN:creatinine ratio was measured at 6 months of age (N = 5 for NZB/W, N = 3 BALB/c). *p < .05; **p < .01 PAM3 vs PBS treated NZB/W mice.

The potential of PAM3 to increase M2 macrophage frequency represents a novel approach to the treatment of autoimmunity. In contrast to agents that non-specifically deplete all types of macrophages (thereby increasing host susceptibility to bacterial infections) PAM3 selectively supports the generation of immunosuppressive macrophages (Fig. 2C).

The patients evaluated in this study were treated with a variety of conventional agents including hydroxychloroquine, prednisone, mycophenolate mofetil (MMF) and azathioprine (AZA) (Table 1). Hydroxychloroquine is an antimalarial that also interferes TLR7 and TLR9 signaling [3,51,52]; prednisone is a glucocorticoid that inhibits T and B cell responses [53]; MMF is an immunosuppressive agent that inhibits lymphocyte proliferation and antibody production [3,54]; while AZA regulates DNA synthesis thereby inhibiting cell proliferation [3]. None of these drugs is known to selectively act on monocytes/macrophages or influence monocyte differentiation.

The relative excess of M1-like macrophages in lupus patients contributes to the increased level of inflammatory cytokines and reduced apoptotic capacity found in that disease [24–27]. When apoptotic cells are not removed by phagocytosis, they leak DAMPS and self-antigens in

a process known as secondary necrosis. These can trigger inflammation and stimulate B cells to produce autoantibodies [55]. Treatment with PAM3 reversed these abnormalities (Fig. 3A), generating M2-like macrophages with good endocytic activity that preferentially secreted the immunosuppressive cytokine IL-10 rather than the inflammatory cytokines IL-12 and TNF α (Fig. 3C–E). Monocytes from patients with active lupus express elevated levels of the co-stimulatory molecule CD40. This improves their ability to activate B cells (which express the CD40 ligand) and supports autoAb production [6,23]. Current findings confirm that lupus monocytes express high levels of CD40. Treatments that support the differentiation of monocytes into M1-like (IFN γ and R848) but not M2-like (PAM3) macrophages correlated with increased CD40 expression (Fig. 2E).

To evaluate the effect of PAM3 *in vivo*, NZB/W mice were treated weekly starting either before or after abnormalities in glomerular function (proteinuria) arose (at 7 and 13 weeks of age, respectively). PAM3 normalized the M1:M2 ratio, inhibited the progression of proteinuria, reduced the BUN:creatinine ratio and prolonged survival (Figs. 4, 6 and 8). Initiating PAM3 treatment at 7 weeks of age maximized its survival benefit. Starting at 13 weeks had less of an effect on the magnitude of proteinuria and survival, indicating that treatment could not reverse pre-existing kidney damage. Histologic evaluation of kidneys from these animals confirmed that glomerular and tubular infiltration were reduced by PAM3 treatment while the frequency of CD206⁺ M2 macrophages rose (Fig. 7). While human lupus is characterized by periods of relative inactivity punctuated by disease flares, glomerulonephritis in NZB/W mice is genetically determined and advances inexorably. Thus, the significant delay in disease progression observed in this murine model may translate into even greater benefit for patients. In that context, treatment with PAM3 significantly altered the immune milieu of NZB/W mice. The production of the pro-inflammatory cytokines IL-12 and TNF α was reduced (Fig. 3C and D; 5B, C). Previous reports showed that neutralizing IL-12 could prevent the development of Th1-mediated autoimmune disease [56]. Similarly, TNF α levels in the sera of lupus patients correlates with disease activity and there are reports that anti-TNF therapy improves disease outcome [29,57] (Figs. 3C and 5C). IL-10 is an immunosuppressive cytokine produced by M2 macrophage. IL-10 impedes APC activation and alleviates disease in NZB/W mice (although its effect in human lupus remains controversial) [29,58,59]. Human monocytes stimulated with PAM3 and splenocytes from PAM3 treated mice produced IL-10 (Figs. 3E and 5D), an effect linked with a reduction in autoAb levels in NZB/W mice (Fig. 5A).

The findings in this report establish that PAM3 supports the preferential generation of M2-like macrophages from lupus monocytes. That activity altered the internal milieu, increasing levels of suppressive versus inflammatory cytokines, reducing autoantibody production, delaying disease progression and significantly prolonging survival. We propose that PAM3 mediated modulation of macrophage differentiation be evaluated for efficacy in human SLE.

5. Conclusions

Current findings support the conclusion that macrophage contribute to the pathogenesis of SLE. Our results demonstrate that the TLR2/1 agonist PAM3 induces monocytes from lupus patients to preferentially differentiate into M2-like macrophages. The same effect is observed when PAM3 is administered to lupus-prone NZB/W mice, culminating in the normalization of the M1:M2 balance. Disease progression is delayed and survival significantly prolonged by weekly PAM3 treatment in this murine model. By regulating macrophage differentiation, PAM3 thus represents a potentially novel approach to the therapy of SLE.

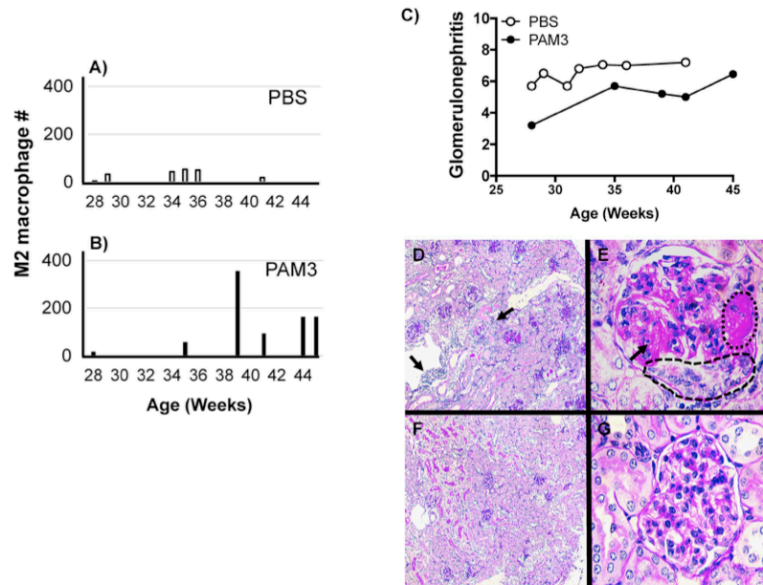


Fig. 7. Effect of PAM3 treatment on kidney pathology and the accumulation of M2 macrophages. NZB/W mice were treated as described in Fig. 6 A,B) The number of M2 macrophages present in their kidneys was monitored over time by immunohistochemical staining of PBS and PAM3 treated mice. C) The severity of glomerulonephritis was evaluated histologically. N = 9 for PBS and 5 for PAM3 treated animals. D-G) Representative PAS stained kidney sections from 28 week old NZB/W mice treated with PBS (D, E) or PAM3 (F, G). Note the increased interstitial inflammation (arrows) in panel D and hyaline deposits (–), cellular crescent formation (–) and karyorrhexis (arrow) in panel E vs the slight increase in endocapillary proliferation in panel G. Panels D, F were 100x and panels E, G were 800x magnification.

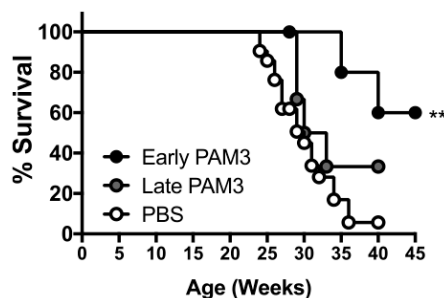


Fig. 8. Effect of PAM3 on survival. Survival was monitored through 45 weeks of age in mice treated with PAM3 starting at 7 or 13 weeks of age (N = 6/group) or PBS treated controls (N = 21). **p = .0037 when compared to PBS group, Log rank test.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2019.01.004>.

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